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Spatial and Temporal Expression of the Ventral Pelvic Skin Aquaporins during Metamorphosis of the Tree Frog, *Hyla Japonica*

T. Hasegawa, Y. Sugawara, M. Suzuki, S. Tanaka
Department of Biology, Faculty of Science, Shizuoka University, Shizuoka 422-8529, Japan

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Abstract. Most adult anurans absorb water through their ventral skin to maintain the proper water balance. We examined spatial and temporal expression of frog (*Hyla japonica*) aquaporins, *Hyla* AQP-h2 and AQP-h3 proteins, in the ventral pelvic skin by using specific antibodies. Immunofluorescence indicates that AQP-h2 and AQP-h3 first appear in the granular cells of the pelvic skin of the tadpoles at Gosner stage 42, and such labeling is seen in later stages as well. These findings were confirmed by Western blot analysis. In addition, Northern blot analysis demonstrated that V_2-type vasotocin (AVT)-receptor mRNA is first expressed at the same stage as are the AQP proteins, which suggests a functional relationship between expression of AQP proteins and AVT receptor. Also, AQP expression in the ventral pelvic skin is consistent with the morphological changes that occur in the skin for adaptation from life in water to that on land.

Key words: Aquaporin — V_2-Type AVT receptor — Immunohistochemistry — Ventral pelvic skin — Frog (*Hyla japonica*)

Introduction

Amphibians were the first vertebrates to emerge from aquatic habitats and modern species have evolved a variety of mechanisms to regulate water homeostasis. To maintain water balance, most adult anurans absorb water through their ventral pelvic skin and reabsorb the appropriate amount of water in the urinary bladder. It is well known that water channels called aquaporins (AQPs) are involved in the regulation of water homeostasis in mammals (Agre, Brown & Nielsen, 1995). Aquaporins form membrane pores that are selectively permeable to water and, isoform-dependently, to certain small solutes, such as glycerol and urea. In mammals, 11 isoforms of AQP have been identified (AQP0–AQP10: King & Agre, 1996; Yamamoto & Sasaki, 1998; Ishibashi Kuwahara & Sasaki, 2000; Hatakeyama et al., 2001). Several AQP isoforms, such as AQP1, display a ubiquitous tissue distribution; others show a tissue-specific expression, for example, AQP0 (originally named major intrinsic protein [MIP] 26) in the eye lens (Gorin et al., 1984) and AQP2 in the apical plasma membrane (Fushimi et al., 1993) and AQP3 in the basolateral membrane (Echevarria et al., 1994; Ishibashi et al., 1994; Ma et al., 1994) of the kidney collecting duct.

Recently, we cloned cDNAs encoding 3 distinct AQPs (AQP-h1, AQP-h2, and AQP-h3) from the ventral pelvic skin of the tree frog, *Hyla japonica* (Tanii et al., 2002; Hasegawa et al., 2003). These proteins consisted of 271, 268, and 271 amino-acid residues, respectively, with each containing 2 Asn-Pro-Ala (NPA) motifs that are conserved in all MIP family members, as well as a cysteine just upstream from the second NPA motif, which confers mercury sensitivity. These proteins have 6 transmembrane regions, with both their N-terminus and C-terminus in the cytoplasm; and they also contain putative sites phosphorylated by protein kinase A, i.e., Ser-262 in AQP-h2 and Ser-255 in AQP-h3. The results of RT-PCR-based studies on the distribution of AQP mRNAs showed that AQP-h1 mRNA was expressed in a wide variety of tissues, and AQP-h2, in several tissues, including antidiuretic hormone (ADH)-dependent tissues, i.e., the ventral pelvic skin, urinary bladder and kidney, but that AQP-h3 mRNA existed only in the ventral pelvic skin (Tanii et al., 2002). An immunofluorescence study (Tanii et al., 2002) showed

Correspondence to: S. Tanaka; email: sbstana@ipc.shizuoka.ac.jp

that the AQP-h3 protein was localized in the pelvic skin, but was not present in the pectoral skin, dorsal skin, kidney, or urinary bladder of the tree frog. In the ventral pelvic skin, the label for AQP-h3 was more intense in the upper layer of the stratum granulosum, and was localized in both the apical and basolateral plasma membranes of the granular cells. These findings indicate that the expression of AQP-h3 in the ventral pelvic skin is essential for the land adaptation of the tree frog. The importance of the ventral pelvic skin for a land adaptation has been pointed out (Jorgensen, 2000; Hillyard, Hoff & Propper, 1998). The label for AQP-h2 was also detected in the same sites as the label for AQP-h3, that is, in the plasma membrane of the granular cells in the adult pelvic skin. In mammals, AQP2 is an ADH-dependent AQP: ADH stimulates the transport of vesicles bearing AQP2 protein from their cytoplasmic pool to the apical plasma membrane via ADH receptors on the kidney granular cells, thereby causing water to enter the cells (Agre et al., 1995, Sasaki, Ishibashi & Marumo 1998). An experiment investigating the responsiveness of the pelvic skins of the tree frog to vasotocin (the nonmammalian vertebrate counterpart of vasopressin or ADH in mammals) showed both *Hyla* AQP-h2 and AQP-h3 to be "ADH"-dependent AQPs (Hasegawa et al., 2003).

Frog skin changes from the larval type to adult type during metamorphosis to adapt to life on land. The ventral pelvic skin of the adult bullfrog is more permeable to water than that of its tadpole (Bentley & Greenwald, 1970). Consequently, we were interested in examining the spatial and temporal expression of AQP-h2 and AQP-h3 proteins in the ventral pelvic skin during metamorphosis. In this study, we examined the ventral pelvic skin of *H. japonica* larvae by immunohistochemistry, using specific antibodies against *Hyla* AQP-h2 and AQP-h3 proteins, and the expression of V_2-type AVT receptor by Northern blot analysis. We found that both AQP proteins and AVT receptor are expressed in the ventral pelvic skin at the climactic stage of metamorphosis.

Materials and Methods

Animals

Larvae of tree frogs (*Hyla japonica*) at various developmental stages were collected from a field near our university in July. They were reared at room temperature in tap water and fed boiled spinach on alternate days. The stages of tadpoles were determined according to Gosner (1960).

Light and Electron Microscopy

The ventral pelvic skins from the tadpoles at various stages were fixed with 2% glutaraldehyde, 2% paraformaldehyde and 0.5% picric acid in 0.1 M cacodylate buffer, pH 7.4, at 4°C for 2 h and postfixed for 1 h at 4°C in 1% osmium tetroxide, reduced with 1.5% potassium ferrocyanide, in the same buffer. After washing with the above buffer, the specimens were dehydrated through a graded ethanol series and embedded in Spurr resin. One micrometer-thick sections were stained with a mixture of 1% toluidine blue and 1% azure II, and ultrathin sections, with uranyl acetate and lead citrate. The ultrathin sections were examined with a Hitachi H-7500 electron microscope at 80 kV.

Immunofluorescence

The ventral pelvic skins, including the underlying dermal and muscular layers, from 5 tadpoles were quickly removed, fixed for 16 h in periodate-lysine-paraformaldehyde (PLP) fixative, dehydrated, and embedded in Paraplast. Sagittal 4 μm sections were cut and mounted on gelatin-coated slides. The deparaffinized sections were rinsed with distilled water and phosphate-buffered saline (PBS). For double labeling of *Hyla* AQP-h2 and *Hyla* AQP-h3, immunofluorescence staining was performed essentially as described previously (Hasegawa et al., 2003). Briefly, the sections were incubated with 1% bovine serum albumin-PBS, a mixture of guinea pig anti-*Hyla* AQP-h2 (1:5,000) serum and rabbit anti-*Hyla* AQP-h3 serum (1:10,000). After having been washed with PBS, the sections were then incubated for 2 h with a mixture of Cy3-labeled affinity-purified donkey anti-guinea pig IgG (Jackson Immunoresearch, West Glove, PA) and FITC-labeled affinity-purified donkey anti-rabbit IgG (Jackson). For nuclear counterstaining, 4′, 6-diamidino-2-phenylindole (DAPI) was included in the secondary antibody solution. The sections were washed with PBS and then mounted in PermaFluor (Immunon, Pittsburgh, PA). To check the specificity of the immunostaining, we performed an absorption test by preincubating the anti-AQP-h2 serum and anti-AQP-h3 serum with the respective peptide (10 μg/ml) used as immunogen. Specimens were examined with an Olympus BX50 microscope equipped with a BX-epifluorescence attachment (Olympus Optical, Tokyo, Japan).

Western Blot Analysis

The ventral pelvic skins taken from 10 tadpoles of the frogs at stages 38 to 46 and from 5 adults were homogenized in cell lysis buffer (50 mM Tris-HCl [pH 8.0], 0.15 M NaCl, 1% Triton X-100, 0.1 mg/ml PMSF, 1 μg/ml aprotinin) and centrifuged in a microcentrifuge for 5 min to remove insoluble materials. Protein amounts were determined with a BCA Protein Assay Kit (Pierce, Rockford, IL). The supernatant protein (10 μg) was denatured at 70°C for 10 min in denaturation buffer comprising 2% SDS, 25 mM Tris-HCl (pH 7.5), 25% glycerol, and 0.005% bromophenol blue, subjected to electrophoresis on a 12% polyacrylamide gel, and then transferred to an Immobilon-P membrane (Millipore, Tokyo, Japan). The proteins in the membrane were reacted sequentially with anti-*Hyla* AQP-h2 (1:10,000) or AQP-h3 (1:20,000) serum, biotinylated goat anti-rabbit IgG (DAKO Japan, Co., Ltd., Kyoto, Japan) or biotinylated donkey anti-guinea pig IgG (Jackson), and streptavidin-conjugated horseradish peroxidase (DAKO). The reaction product on the membrane was visualized by using an ECL Western blot detection kit (Amersham Pharmacia Biotech, Buckingamshire, UK).

Cloning of the Tree Frog V_2-Type AVT Receptor

Purified DNA from a tree frog ventral pelvic skin cDNA library (Tanii et al., 2002) was amplified by the polymerase chain reaction (PCR) in a thermal cycler (ASTEC, Fukuoka, Japan). The procedure for the PCR amplification was an initial denaturation step of

95°C for 5 min followed by denaturation (94°C, 90 s), annealing (50°C, 90 s), and extension (72°C, 150 s) for 30 cycles. Primers 1 and 3 (Sawady Technology, Tokyo, Japan) were designed based on the known regions of V_2-type AVT receptor from the tree frog (Kohno, Kamishima & Iguchi, 2003). Nested PCR amplification was further performed by using primers 2 and 3. The sequences of sense (primers 1, 2) and antisense (primer 3) primers were as follows: AVT-R primer 1,5′-GTGGTGGCTTTCTTCCAAGT-3′; AVT-R primer 2,5′-TGACTTGGATAACACTGGCG-3′; and AVT-R primer 3,5′-ACAGCTGTTCAGACTGGCTA-3′. The amplified PCR products were electrophoresed on a 2% agarose gel, and the 356-bp fragment (the expected size based on the known tree frog AVT-R cDNA sequence) was subcloned directly into the pGEM-3Z vector (Promega, Madison, WI) and sequenced. Digoxigenin (DIG)-labeled antisense cRNA probe was prepared from the partial coding region of the AVT receptor by in vitro transcription, as described previously (Saito et al., 2002). We synthesized cRNA probes with sequences identical to those of the PCR products described above by using a DIG-High Prime kit (Roche Mol. Biochem., Meylan, France).

Northern Blot Analysis for the V_2-Type AVT Receptor

Total RNA was isolated from 50 mg of the ventral pelvic skins of larvae at stages 38–45, and the pelvic and dorsal skins of the adult frogs by using TRIZOL reagent. Ten micrograms of the total RNA from each sample was electrophoresed on a denatured gel containing 1% agarose and 2 M formaldehyde and blotted onto a nylon membrane (Roche). The RNAs were fixed on the membrane by UV cross-linking.

The membrane was prehybridized for 1 h at 65°C in prehybridization solution. Hybridization with the DIG-labeled cRNA probe of the AVT receptor was performed for 15 h at 65°C. The membrane was washed at room temperature once for 20 min in 2 × saline-sodium citrate (SSC) containing 0.1% SDS and twice at 65°C, for 30 min each time, in 0.2 × SSC containing 0.1% SDS. After a blocking step, the membrane was incubated with alkaline phosphatase-labeled anti-DIG antibody (Roche) for 30 min at room temperature, reacted with CSPD for 5 min, and then exposed to Hyperfilm-ECL (Amersham).

Results

Morphological Changes in the Ventral Pelvic Skin and Expression of AQP Proteins during Metamorphosis

The larval pelvic skin at Gosner stage 37 is composed of a single layer of apical cells, with skein cells and basal cells in the underlayer (Fig. 1*a*). Electron microscopical observation revealed the presence of tight junctions between the apical cells and granular-like structures in the cytoplasm beneath the apical plasma membrane, as well as many mitochondria and lysosomes near the nucleus (Fig. 1*b*). The skein cells were connected to the basal lamina by means of developed hemi-desmosomes, where keratin fibers, called the figure of Eberth, were seen. At the climactic stage, especially stage 42, the skin was organized into the same 3 layers as found in adulthood: the stratum corneum, the granulosa, and the germinativum

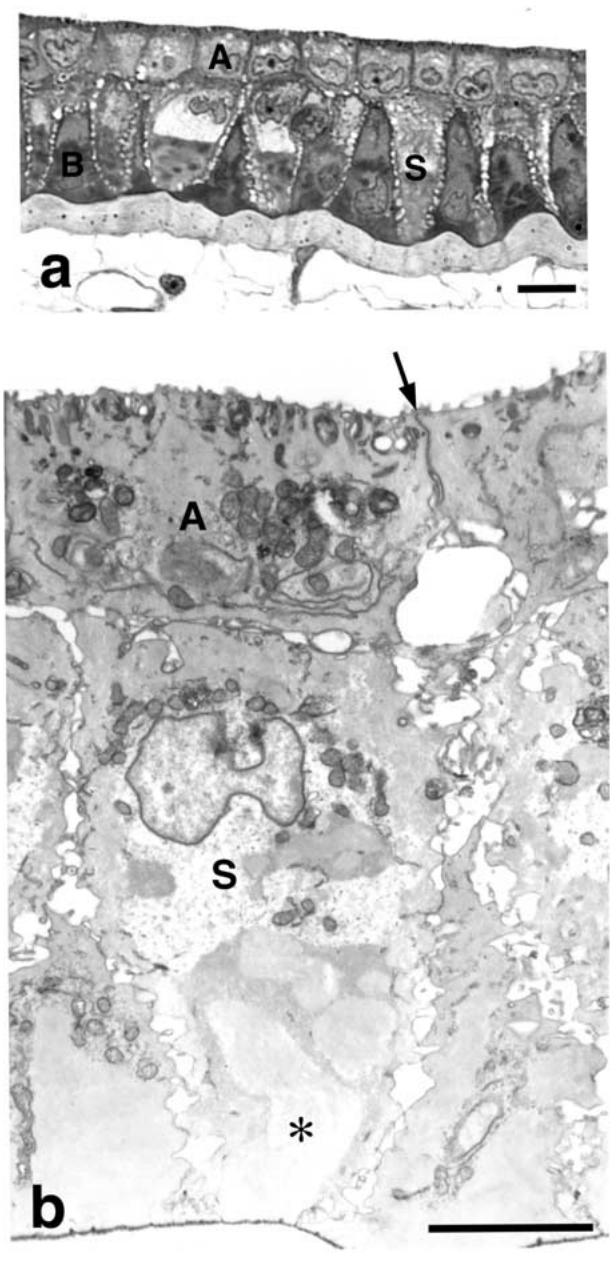

Fig. 1. Light (*a*) and electron (*b*) micrographs showing sections of ventral pelvic skin at Gosner stage 37. Larval skin is composed of a single layer of apical cells (*A*), with skein cells (*S*) and basal cells (*B*) in the underlayer. The *arrow* and *asterisk* in *b* indicate a tight junction and the figure of Eberth, respectively. Bar: *a*, 10 μm; *b*, 5 μm.

(Fig. 2*a*). Mitochondria-rich cells were often observed in the epithelium. However, apical cells and skein cells characteristic in larval life were not observed. By electron microscopy, several granules and keratin fibers were seen in the granular cells, just as in the adult skin (Fig. 2*b*, *c*).

When immunofluorescence staining was performed on the ventral pelvic skin according to metamorphic stages, no immunopositive reactions for

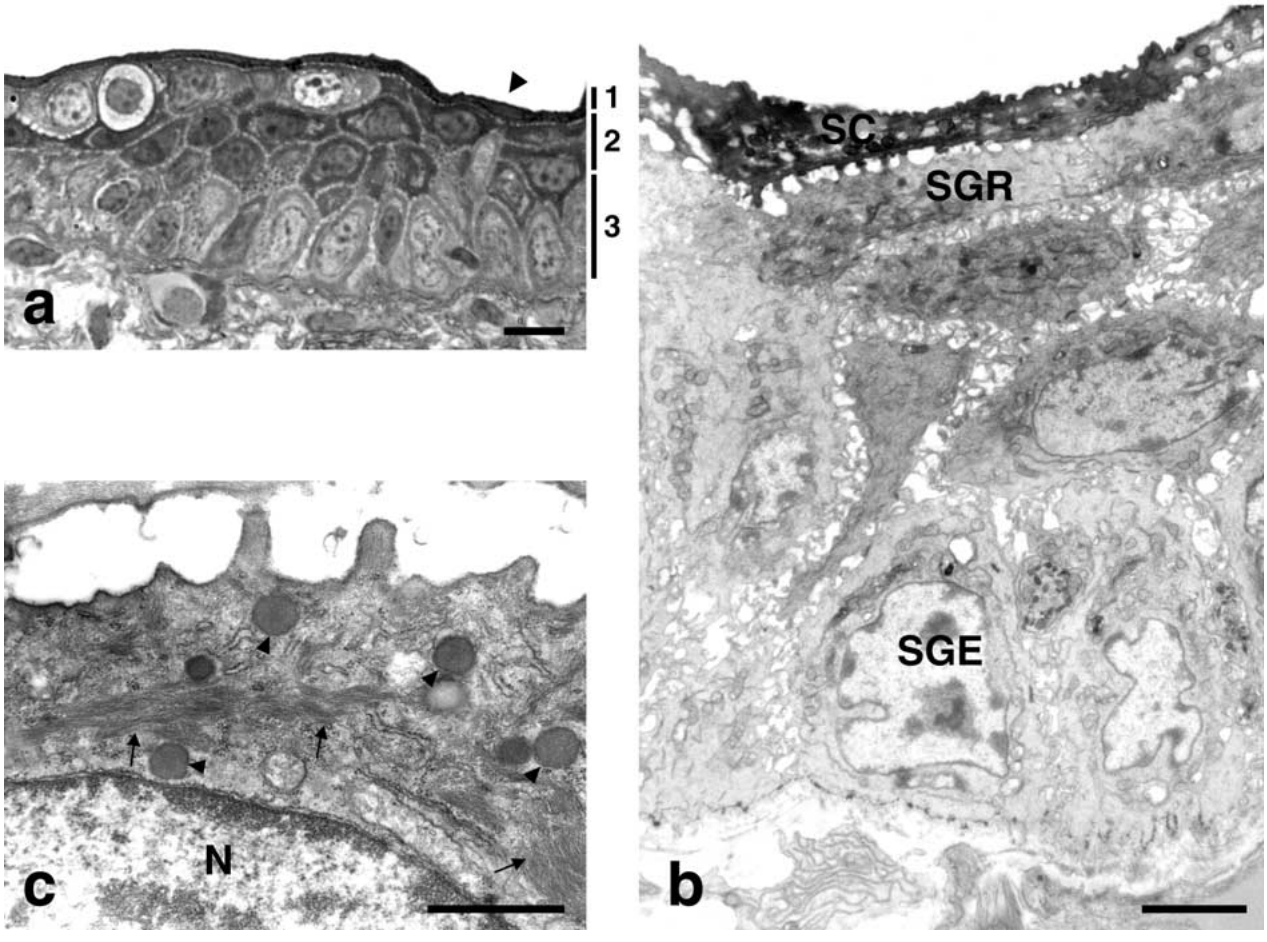

Fig. 2. Light (*a*) and electron (*b, c*) micrographs showing specimens of ventral pelvic skin at Gosner stage 42. (*a*) The skin is organized into 3 layers: the stratum corneum (*1*), the granulosa (*2*), and the germinativum (*3*), comprising granular cells and mitochondria-rich cells (*arrowhead*). (*b*) An electron micrograph showing the pelvic skin at low magnification. *SC*, stratum corneum; *SGR*, stratum granulosum; *SGE*, stratum germinativum. (*c*) An electron micrograph showing part of a granular cell. *Arrowheads*, granules; *N*, nucleus. *Bar*: *a*, 10 μm, *b*, 5 μm *c*, 1 μm.

AQP-h2 and AQP-h3 were observed during premetamorphic and prometamorphic stages (Fig. 3). At the climactic stages, especially stages 42 and 43, granular cells immunopositive for both AQP-h2 and AQP-h3 were detected (Fig. 3 *a3–c3*, *a4–c4*). The positive labelings were found in the basolateral plasma membrane of the granular cells in 1 or 2 sublayers of the stratum granulosa, located just beneath the stratum corneum. In the absorption test, the immunoreaction obtained with either antiserum was completely eliminated when 0.01 mg/ml of the respective antigen peptide was used as an adsorbent (Fig. 4).

In the Western blot analysis of the pelvic skin, positive bands were first found at stage 42 (Fig. 5). Main bands of 29 kDa for AQP-h2 and 29 kDa for AQP-h3 were consistently detected from stage 42 to the completion of metamorphosis, although the intensity of the AQP-h2 band was weaker than that of the AQP-h3 one, especially in the case of stage 42.

Northern Blot Analysis of V_2-Type AVT Receptor mRNA

Northern blot analysis of AVT mRNAs isolated from the ventral pelvic skin of tadpoles and the pelvic and dorsal skin of adult tree frogs was performed by using a DIG-labeled AVT receptor cRNA as a probe. A single strong band was detected at a position corresponding to approximately 2.4 kb with the ventral pelvic skin samples, whereas no positive signal was obtained from the dorsal skin sample (Fig. 6).

The content of the AVT receptor mRNA abruptly increased at stage 42 and then was maintained at relatively high levels afterward.

Discussion

The present study demonstrates that both AQP-h2 and AQP-h3 proteins appear at Gosner stage 42, at

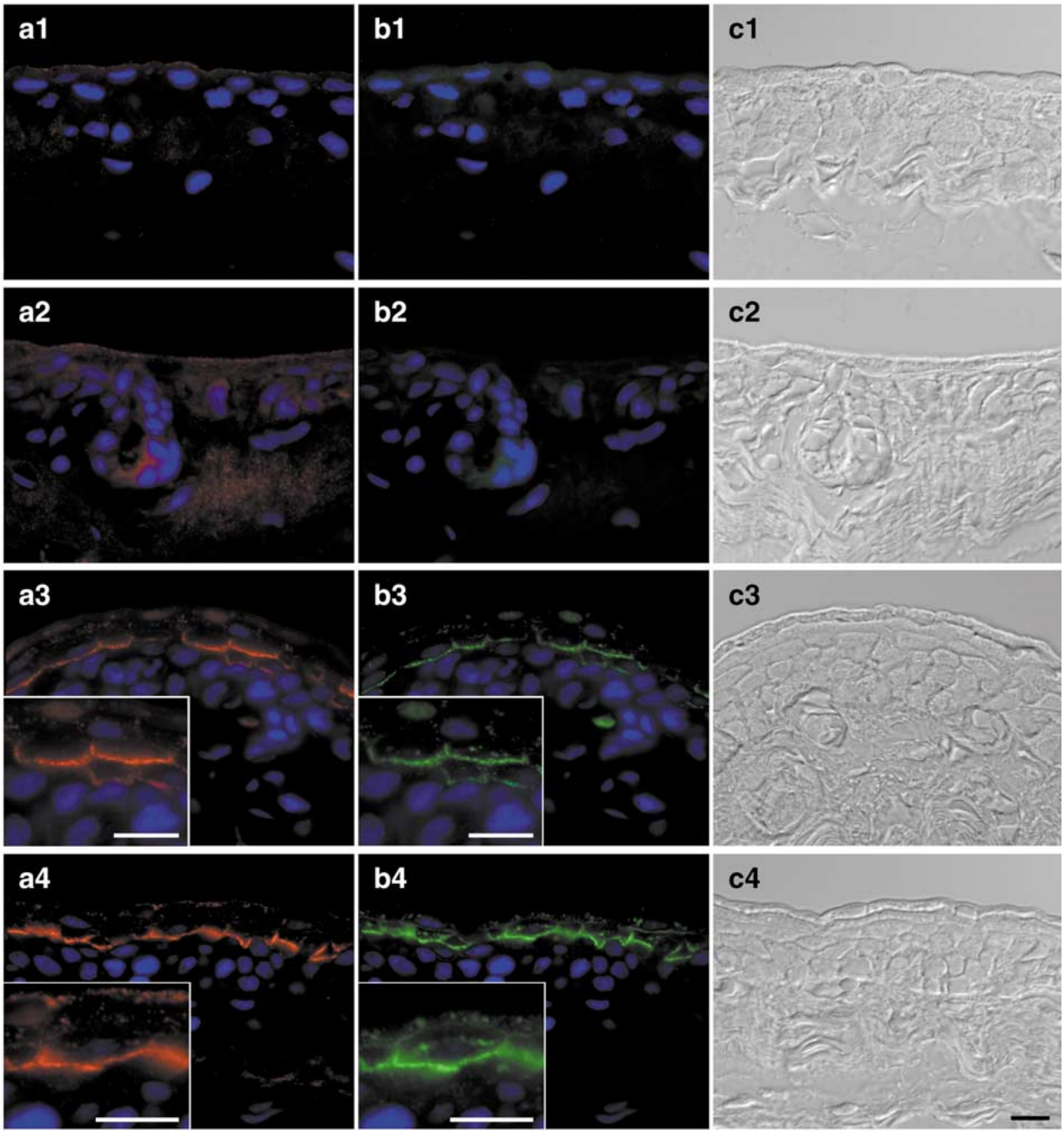

Fig. 3. Double-immunofluorescence micrographs showing developmental stages of ventral pelvic skin labeled for AQP-h2 (*a*, *red*) and AQP-h3 (*b*, green); *c*, Nomarski image for *a–b*. *a1–c1*, Gosner stage 40; *a2–c2*, stage 41; *a3–c3*, stage 42; *a4–c4*, stage 43. Insets in a3, b3, a4, and b4 show higher magnification of each of the figures. *Bars*: *a–c*, 10 μm, inset, 10 μm.

which stage forelimbs appear in the tadpole and the transformation of the larval skin into the adult type is completed. This finding suggests that both AQP-h2 and AQP-h3 are important for the land-dwelling adult frog to be able to absorb water from its ventral pelvic skins. In the present morphological observation, we show that the larval skin before stage 37 consists of apical cells, skein cells, and basal cells. On the other hand, our immunofluorescence study demonstrates that the larval ventral pelvic skin does not express any AQP proteins, but that both AQP-h2 and AQP-h3 appear at stage 42, when the ventral skin has nearly become transformed into the adult type. The ventral skin of the tadpoles at the metamorphic climax is composed of granular cells and mitochondria-rich cells similar to those seen in the adult skin. Moreover, the present study demonstrates that the immunosignals indicating AQP-h2 and AQP-h3 come from the

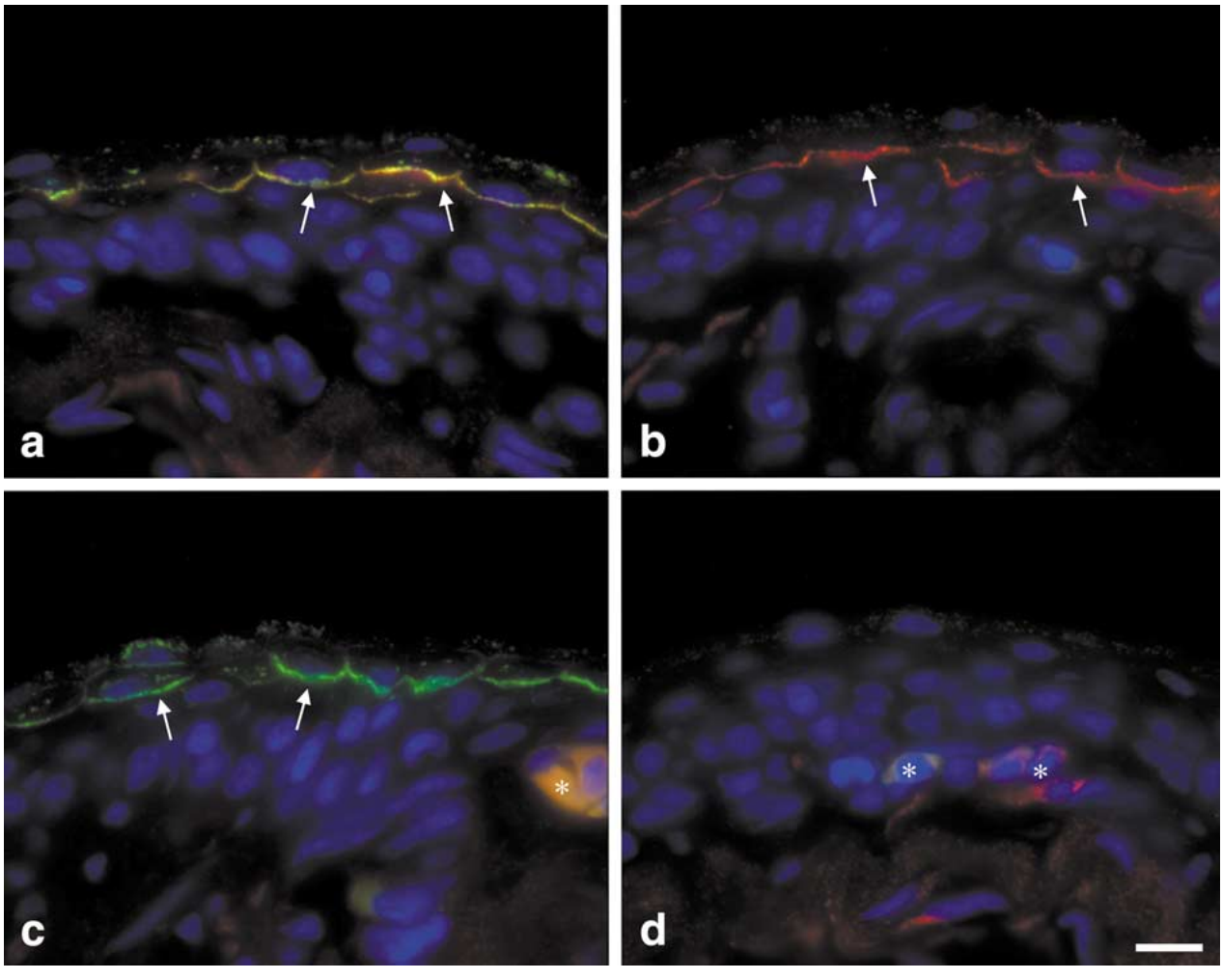

Fig. 4. The specificity of immunolabeling. An immunofluorescence image shows localization of AQP-h2 and AQP-h3 proteins (*a*) in the ventral pelvic skin of stage 42 tadpole. Labeling for only AQP-h2 (*b*) or AQP-h3 (*c*) is detected when anti-AQP-h2 was preabsorbed with AQP-h3 peptide or when anti-AQP-h3 was preabsorbed with AQP-h2. No labeling is seen when the antisera were preabsorbed with both AQP-h2 and AQP-h3 peptides (*d*). *Arrows* and *asterisks* indicate immunopositive labeling of the granular cells and red blood cells, respectively. *Bar*: *a–d* = 10 μm.

basolateral plasma membrane of the granular cells in 1 or 2 layers of the stratum granulosum, located just beneath the stratum corneum. During the transformation of the larval skin into the adult type, the epithelial cells and skein cells are lost as a consequence of the repeated division of the basal cells and their differentiation into granular cells (Robinson & Heintzelman, 1987). During this developmental transformation of basal cells into granular cells, the AQP proteins may be expressed first. In addition, the present study showed that this pattern of immunolabeling constantly continued into adulthood.

Using the doule-immunofluorescence method, we revealed that the distribution of AQP-h2 and AQP-h3 proteins in the ventral pelvic skin show a similar pattern. This labeling pattern may raise doubts as to whether the labeling is real or an artifact, but the specificity of labeling was confirmed by the absorption test with the immunogen peptides. Therefore, we assume that the immunolabeling pattern is real. The high resolution power of immunoelectron microscopy will provide further information about the exact localization of both of the AQPs.

Our Western blot analysis confirmed these immunofluorescence data. Previous studies showed that N-glycosylation sites were present at Asn-124 in AQP-h2 and at Asn-124 and Asn-125 in AQP-h3 (Tanii et al., 2002; Hasegawa et al., 2003). However, we detected only a non-glycosylated 29-kDa band in the tadpole pelvic skin, although both non-glycosylated and glycosylated forms were observed in the adult pelvic skin. This discrepancy may be due to active synthesis of AQP proteins at early development.

It was earlier reported that the pelvic region was the site richest in V_2-type AVT receptors in the ventral pelvic skin of the adult tree frog, with fewer in the

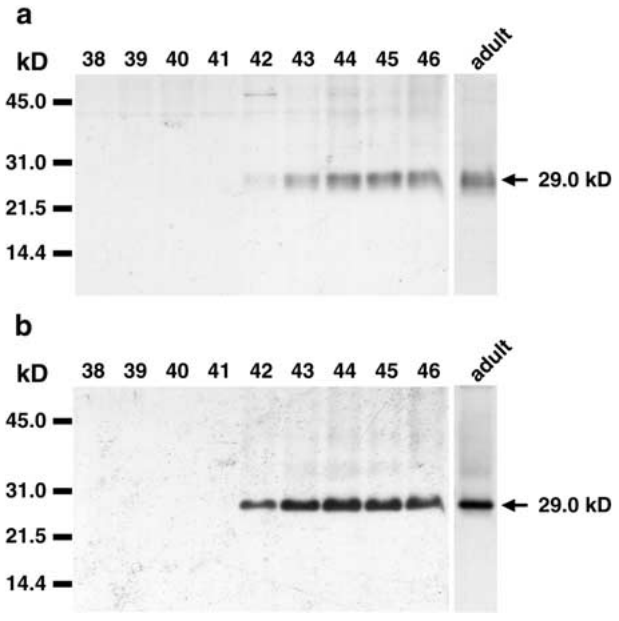

Fig. 5. Expression of AQP-h2 and AQP-h3 proteins in the ventral pelvic skin during metamorphosis. Ten micrograms of protein of tissue homogenate prepared from the pelvic skin were applied to SDS-PAGE (12% acrylamide), electrophoresed, and subjected to immunoblotting with anti-AQP-h2 (*a*) and with AQP-h3 (*b*). *Arrows* indicate immunopositive band (29 kD).

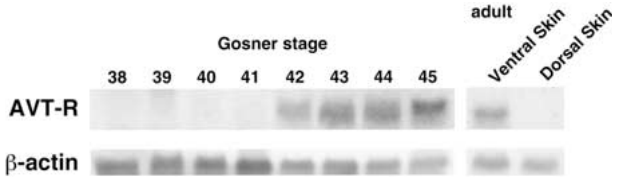

Fig. 6. Northern blot analysis of V_2-type vasotocin-receptor mRNA during metamorphosis and in the adult skin. Total RNA (10 μg) extracted from the ventral pelvic skin of tadpoles and from the pelvic and dorsal skin of adult frogs was electrophoresed in 2% agarose gel containing 2 M formaldehyde. V_2-Type vasotocin-receptor mRNA of approximately 2.4 kb was expressed in the pelvic skin samples only.

dorsal skin (Kohno, Kamishima & Iguchi, 2003). However, there was heretofore no report on the ontogenic expression of the AVT receptor in the pelvic skin. In the present study, we demonstrate that V_2-type AVT receptor mRNA is first expressed at stage 42, which is also the stage at which the AQP proteins are first expressed. The expression of AQP proteins establishes a mechanism for the increased ability to absorb water across the skin, which occurs in parallel with the change in the structure of the ventral pelvic skin. Thus the physiological differentiation and the structural differentiation appear to occur simultaneously.

The present study demonstrates that both AQP proteins and the AVT receptor are expressed in the ventral pelvic skin of the climactic tadpoles. However, we doubt that these tadpoles have the same ability for water uptake as the adults, because Bentley and Greenwald (1970) reported that the skin of adult bullfrogs was approximately 4 times more permeable to water than that of their tadpole. In adult tree frogs, we previously found that both AQP-h2 and AQP-h3 proteins were translocated from the cytoplasmic pool to the apical plasma membrane in the pelvic granular cells upon stimulation by AVT, thereby inducing water uptake in the body (Hasegawa et al., 2003). On the other hand, a study conducted years ago showed that water uptake increased when tadpoles at climactic stages were injected with AVT, but that the intensity of the uptake was only 1.5 times greater than that of the controls (Bentley & Greenwald, 1970). Consequently, the translocation of AQP proteins induced by AVT may not be fully developed in the climactic tadpoles. In the near future, it will be necessary to clarify this issue.

Hillyard, Zeiske and Van Driessche (1982) showed that Na^+ transport across the bullfrog ventral pelvic skin was low during the early larval period, but increased at the climactic stages. Moreover, morphological changes in the skin are known to be induced by thyroid hormone. Using an in vitro culture system, Takata, Yai and Takayama-Arita (1995) found that corticoid, but not thyroid hormone regulated Na^+ transport across the larval bullfrog skin. It will be necessary to clarify the induction mechanism of AQP expression.

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