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Molecular and cellular characterization of a new aquaporin, AQP-x5, specifically expressed in the small granular glands of *Xenopus* skin

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Summary

A new toad aquaporin (AQP) cDNA was cloned from a cDNA library constructed from the ventral skin of *Xenopus laevis*. This AQP (*Xenopus* AQP-x5) consisted of 273 amino acid residues with a high sequence homology to mammalian AQP5. The predicted amino acid sequence contained the two conserved Asn-Pro-Ala motifs found in all major intrinsic protein (MIP) family members and six putative transmembrane domains. The sequence also contained a mercurial-sensitive cysteine and a putative phosphorylation motif site for protein kinase A at Ser-257. The swelling assay using *Xenopus* oocytes revealed that AQP-x5 facilitated water permeability. Expression of AQP-x5 mRNA was restricted to the skin, brain, lungs and testes. Immunofluorescence and immunoelectron microscopical studies using an anti-peptide antibody (ST-156) against the C-terminal region of the AQP-x5 protein revealed the presence of immunopositive cells in the skin, with the label predominately localized in the apical plasma membrane of the secretory cells of the small granular glands. These glands are unique both in being close to the epidermal layer of the skin and in containing mitochondria-rich cells with vacuolar H^+-ATPase dispersed among its secretory cells. Results from immunohistochemical experiments on the mucous or seromucous glands of several other anurans verified this result. We conclude that the presence of AQP-x5 in the apical plasma membrane of the small granular glands suggests its involvement in water secretion from the skins. The physiological roles of the AQP-x5 protein in the small or mucous glands are discussed.

Key words: aquaporin, water channel, cDNA cloning, exocrine gland, skin, immunocytochemistry, *Xenopus laevis.*

Introduction

Since the discovery of CHIP28 as a cell-membrane water channel by Preston and Agre (Preston and Agre, 1991), many different water-channel proteins, called aquaporins (AQPs), have been identified in various cells of mammals and lower organisms (Ishibashi et al., 2000; Park and Saier, Jr, 1996). Aquaporins form membrane pores that are selectively permeable to water, and isoform-dependently, to certain small solutes, such as glycerol and urea. In mammals, 13 isoforms of AQPs (AQP0-AQP12) have been identified and characterized by the cloning and sequencing of their cDNA (Ishibashi et al., 2000; Takata et al., 2004). Among mammalian AQPs, AQP5 is expressed specifically in exocrine glands, including the salivary gland, the lacrimal gland and the respiratory tissue, and plays a role in the secretion of water (Matsuzaki et al., 1999; Raina et al., 1995).

We have recently cloned cDNAs encoding three distinct AQPs (AQP-h1, AQP-h2 and AQP-h3) from the ventral pelvic skin of the tree frog, *Hyla japonica* (Hasegawa et al., 2003; Tanii et al., 2002). AQP-h1 was found to be homologous to mammalian AQP1 and showed a ubiquitous tissue distribution. AQP-h2 protein was expressed in the ventral pelvic skin and urinary bladder but not in the kidney, whereas AQP-h3 displayed a specific distribution that was restricted to the ventral pelvic skin. However, antibodies against these frog AQP proteins did not recognize any cellular components of the skin glands. Therefore, we considered that some kind of AQP is expressed in the skin glands of amphibians because the skin of anuran amphibians is always kept moist by watery secretion from these glands.

The skin glands of amphibians are broadly classified into two types: the granular gland (also called the serous gland or poison gland) and the mucous gland (Noble and Noble, 1944; Vanable, Jr, 1964). The granular glands are involved in protecting the amphibian's body by producing either poisonous or irritating secretions in response to violent stimulation (Myers and Daly, 1983) or biogenic amines and a large variety of biologically active substances (Erspamer, 1994). The mucous glands, however, produce only a watery secretion containing Na^+ and Cl^-, the purpose of which is to keep the skin moist (Campantico et al., 1978). In a series of experiments with *Xenopus* skin,

Fujikura et al. (Fujikura et al., 1988) described two additional glands: the small granular (granulated) gland and the NP gland. The former is located close to the epidermal layer of the skin and is the smallest of the skin glands. It is also characterized by having mitochondria-rich (MR) cells among the secretory cells. The NP gland can only be found in the nuptial pad of the male forelimb.

We report here the identification of a new member of the amphibian AQP family, AQP-x5, which we have isolated from the skin of *Xenopus*. AQP-x5 shows a high amino acid sequence homology to mammalian AQP5 and is predominately expressed in the apical plasma membrane of secretory cells in the small granular gland.

Materials and methods

Animals

Adult toads (*Xenopus laevis* Daudin) were kept under laboratory conditions and fed trout food on a regular regimen. The ventral pelvic skin was removed from anesthetized animals (MS 222; Nacalai tesque, Kyoto, Japan) for processing and subsequent use in cDNA cloning experiments. The animals were then killed, and the ventral skin, dorsal skin, urinary bladder, kidneys, brain, heart, lungs, liver, stomach, small intestine, large intestine, testes, ovaries and blood cells were removed for analysis by reverse transcription (RT)-PCR, western blot and immunocytochemistry. For comparative purposes, adult animals of *Rana japonica*, *R. catesbeiana*, *R. nigromaculata*, *Hyla japonica* and *Bufo marinus*, purchased from a commercial dealer, were killed and their tissues used for the immunofluorescence experiments. All animal experiments were carried out in compliance with the Guide for Care and Use of Laboratory Animals of Shizuoka University.

Construction of the toad ventral skin cDNA library

Total RNA was prepared from 0.5 g of toad skin and poly(A)$^+$ RNA from the total RNA, a λZAP DNA library (9.6×10^6 pfu μg^{-1} of vector arms) was constructed, as previously described by Tanii et al. (Tanii et al., 2002).

Oligonucleotide primers for the PCR analysis

Degenerate primers for the original amplification of frog AQP fragments were designed based on the amino acid sequences around the two conserved NPA (asparagine-proline-alanine) boxes of the major intrinsic protein (MIP) family aquaporins (Agre et al., l995). The following primers were commercially synthesized (Gibco-BRL, Rockville, Md., USA): P1 (sense), 5′-AGCGGGG(CG)(CT)CAC(AC)T-(CT)AACCC-3′; P2 (antisense), 5′-GG(AT)CC(AG)A(CT)-CCA(AG)AAGA(CT)CCA-3′; P3 (antisense), 5′-A(AG)-(AG)GA(CG)C(GT)(GT)GC(AT)GG(AG)TTCAT-3′.

RT–PCR amplification and sequence analysis

The skin poly(A)$^+$ RNA (0.5 μg) was reverse-transcribed, and partial sequences of AQP-x5 cDNA were amplified by PCR, as described by Tanii et al. (Tanii et al., 2002). Amplified fragments were subcloned into pGEM-3Z vectors (Promega, Madison, WI, USA) and sequenced using an Aloka DNA sequencing system [Model Lic-4200L(S); Aloka, Japan]. The sequence of one clone corresponded to that of rat AQP5, and was subsequently designated AQP-x5.

Screening of the toad skin cDNA library

A DNA probe, obtained from the first PCR product, was synthesized using a digoxigenin (DIG)-High Prime kit (Roche Molecular Biochemicals, Meylan, France) and used to screen the pelvic skin cDNA library, as previously described (Tanii et al., 2002).

RT–PCR of Xenopus tissues

The tissue expression of AQP-x5 mRNA was analyzed by RT–PCR. TRIZOL reagent (Invitrogen, Carlsbad, CA, USA) was used to prepare total RNA from various *Xenopus* tissues. Total RNA (20 μg) was first treated with DNase I (4 i.u.; Takara, Kyoto, Japan), following which a 10 μg sample of the total RNA product was reverse transcribed at 42°C for 1 h and then at 52°C for 30 min in 20 μl of reaction buffer containing 1 mmol l^{-1} each of dNTP, 9.9 i.u. RAV-2 reverse transcriptase (Takara), 20 i.u. RNase inhibitor (Toyobo, Osaka, Japan), and 7.5 mmol l^{-1} oligo(dT)$_{19}$ primer (Gibco-BRL). RT–PCR was performed basically by the same method described above, using the homologous primers: P3 (sense), 5′-CAGTATCC-TGTTACTCTGTC-3′ (1106–1125 b); P4 (antisense), 5′-ATCTGCCTCTTAATTGACCG-3′ (1482–1501 b). The RT–PCR products were analyzed on a 2% agarose gel containing ethidium bromide (EtBr; 0.5 μg ml^{-1}). Marker 6 (λ/*Sty*I digest; Wako Pure Chemicals, Osaka, Japan) was used as the molecular mass marker.

Antibody

An oligopeptide corresponding to the C-terminal amino acids 261-274 (ST-156: LYSAHPLPKVIDKF) of the *Xenopus* AQP-x5, with an amino-terminal cysteine residue, was synthesized with a Model 433A synthesizer (PE-Applied Biosystems, Foster City, CA, USA). The crude peptide was purified by reverse-phase high-performance liquid chromatography with a 0%–60% linear gradient of CH_3CN in 0.1% trifluoroacetic acid. Purification of the peptide was confirmed by measuring its molecular mass by mass spectrometry. The antibody was raised in a guinea pig immunized with the ST-156 peptide coupled to keyhole limpet hemocyanin (Pierce, Rockford, IL, USA), as described previously (Tanaka and Kurosumi, 1992). The rabbit anti-bullfrog V-ATPase E-subunit had been generated and characterized previously (Yajima et al., 2005): the antiserum was raised against a synthetic peptide (ST-173:: VALFGANANRKFLD) covering amino acids 213–226 of bullfrog V-ATPase E-subunit (DDBJ/EMBL/GenBank accession no. AB250092).

Osmotic water permeability of oocytes

cRNAs were prepared from linearized pBK-CMV phagemid vectors containing the entire open reading frame (ORF) of

AQP-x5 by digestion with *Xho*I (Takara) and transcribed/capped with T_3 RNA polymerase (mCAP™ RNA capping kit, Stratagene, La Jolla, CA, USA). Stage V and VI *Xenopus* oocytes were defolliculated using collagenase (1 mg ml^{-1}; Roche) and microinjected with either cRNAs (50 ng) or water. After a 3-day incubation in Barth's buffer at 18°C, the oocytes were transferred from 200 mOsm to 70 mOsm Barth's buffer, and the osmotically elicited increase in volume was monitored at 24°C under an Olympus BX50 microscope with a 4× magnification objective lens and a CCD camera connected to a computer. The coefficient of osmotic water permeability (Pf) was calculated from the initial slope of oocyte swelling according to accepted methodology (Fushimi et al., 1993; Zhang et al., 1990). In some experiments, $HgCl_2$ was added to a final concentration of 0.3 mmol l^{-1} for 10 min. To confirm whether AQP-x5 protein was expressed in *Xenopus* oocytes after the injection of AQP-x5 cRNA, we analyzed AQP-x5 cRNA-injected or water-injected oocytes by western blot analysis and immunostaining as described below.

Histology

Xenopus ventral skins were fixed by immersion in Bouin's solution for 2 days, dehydrated, embedded in Paraplast, and cut into thin (4 μm) sections. The deparaffinized sections were stained with Mallory's triple stain, dehydrated with a graded ethanol series, and mounted in Enthellan (Merck, Darmstadt, Germany).

Immunofluorescence

The ventral or dorsal skins of the anurans were fixed overnight at 4°C in periodate-lysine-paraformaldehyde (PLP) fixative, dehydrated, and embedded in Paraplast. Thin (4 μm) sections were cut and mounted on gelatin-coated slides, deparaffinized, rinsed with distilled water and phosphate-buffered saline (PBS). For single labeling of AQP-x5 protein, immunofluorescence staining was performed essentially as described previously (Tanaka et al., 1997). The sections were sequentially incubated with 1% bovine serum albumin–PBS, guinea pig anti-AQP-x5 serum (ST-156; 1:2000), and Alexa Fluor 488-labeled goat anti-guinea pig IgG (1:200; Molecular probes, Eugene, OR, USA). For nuclear counterstaining, 4′, 6-diamidino-2-phenylindole (DAPI) was included in the secondary antibody solution. The sections were finally washed with PBS and then mounted in PermaFluor (Immunon, Pittsburgh, PA, USA). The specificity of the immunostaining was checked using an absorption test by preincubating the anti-AQP-x5 antiserum with the antigen peptide (10 μg ml^{-1}). For double-immunofluorescence staining for AQP-x5 and V-ATPase, sections were first incubated with a mixture of guinea pig anti-AQP-x5 (1:2000) and rabbit anti-V-ATPase E-subunit (1:4000) (Yajima et al., 2005) and then reacted with a mixture of Alexa Fluor 488-labeled goat anti-guinea pig IgG (1:200), indocarbocyanine (Cy3)-labeled donkey anti-rabbit IgG (ST-173; 1:400; Jackson Immunoresearch, West Grove, PA, USA) and DAPI. Specimens were examined with an Olympus BX50 microscope equipped with a BX-epifluorescence attachment (Olympus Optical, Tokyo, Japan).

Western blot analysis

The AQP-x5 cRNA-injected or water-injected oocytes were homogenized in cell lysis buffer (50 mmol l^{-1} Tris–HCl, pH 8.0, 0.15 mol l^{-1} NaCl, 1% Triton X-100, 0.1 mg ml^{-1} PMSF, 1 μg ml^{-1} aprotinin) and centrifuged in a microcentrifuge for 10 min to remove insoluble materials. The proteins were determined using a BCA Protein Assay kit (Pierce). The supernatant protein (5 μg) was denatured at 70°C for 10 min in denaturation buffer [2% sodium dodecyl sulfate (SDS), 25 mmol l^{-1} Tris–HCl, pH 7.5, 25% glycerol, 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 on the membrane were reacted sequentially with anti-AQP-x5 serum (ST-156), biotinylated goat anti-guinea pig IgG (DAKO Japan, Kyoto, Japan), and streptavidin-conjugated horseradish peroxidase (DAKO Japan). The reaction product on the membrane was visualized using an ECL western blot detection kit (Amersham Pharmacia Biotech, Buckinghamshire, UK). To check the specificity of the immunoreaction, we performed an absorption test by preincubating anti-AQP-x5 (ST-156) with the antigen peptide (10 μg ml^{-1}).

Conventional electron and immunoelectron microscopy

For conventional electron microscopy, pieces of *Xenopus* skin (~1 mm^3) were fixed with a mixture of 2% paraformaldehyde and 2% glutaraldehyde in 0.1 mol l^{-1} cacodylate buffer, pH 7.4, for 2 h at 4°C and postfixed for 1 h at 4°C in the same buffer. They were dehydrated through a graded ethanol series and embedded in an Epon/Araldite mixture. The ultrathin sections were stained, first with uranyl acetate, then with lead citrate. For immunoelectron microscopy, similar tissues were fixed with PLP for 16 h at 4°C, dehydrated through a graded ethanol series, and embedded in LR White (London Resin, Basingstoke, UK). Pale-gold ultrathin sections were cut with a Reichert Ultracut-E microtome (Richert-Jung, Vienna, Austria) equipped with a diamond knife and mounted on nickel grids. The sections were immunolabeled with guinea pig anti-*Xenopus* AQP-x5 serum (1:4000) followed by goat anti-guinea pig IgG conjugated with 10 nm gold particles (BioCell, Cardiff, UK). As a control, before being examined, several sections were labeled using antiserum preabsorbed with the corresponding antigen peptide at a final concentration of 10 μg ml^{-1}, at 4°C for 16 h. After immunolabeling, the sections were stained with a mixture of uranyl acetate and methyl cellulose according to a published protocol (Roth et al., 1990) and then examined with a Hitachi 7500 electron microscope at 80 kV.

Results

Cloning and sequencing of AQP-x5

Fig. 1 shows the full cDNA sequence of *Xenopus* AQP-x5 and the deduced amino acid sequence. The cDNA consisted of a 63-bp 5′-untranslated region (UTR) and a 1841-bp 3′-UTR followed by a poly(A) tail. The ORF encoded a protein of 273

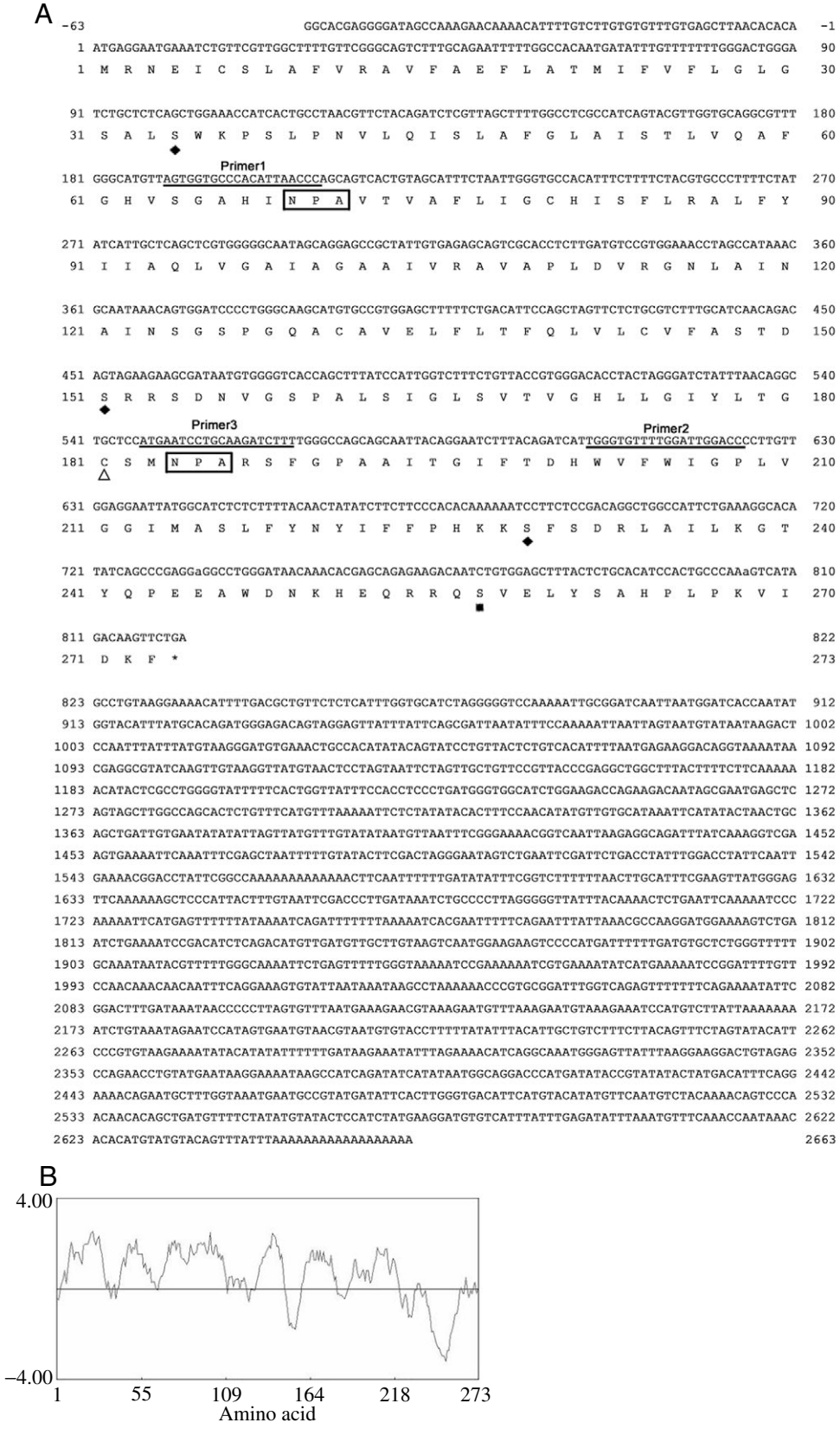

Fig. 1. (A) Nucleotide and deduced amino acid sequence of *Xenopus* AQP-x5 cDNA. The predicted amino acid is shown below the nucleotide sequence. The asterisk indicates the terminal codon. NPA motifs and polyadenylation signal regions are outlined. The square, diamonds, and open triangles indicate phosphorylation sites for protein kinase A, protein kinase C and mercurial-inhibition sites, respectively. (B) Kyte-Doolittle hydropathy profile (window 11) of the deduced AQP-x5 amino acid sequence.

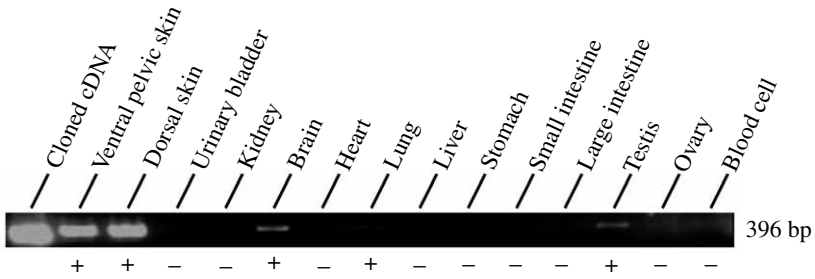

Fig. 2. RT–PCR of *Xenopus* tissue extracts. RT–PCR products using primers as described in the Materials and methods were separated on a 2% agarose gel and stained with ethidium bromide. +, mRNA present; –, mRNA absent.

amino acids with a molecular mass calculated to be 29 443 Da. Hydropathy analysis predicted six transmembrane regions with an N terminus and a C terminus localized in the cytoplasm, which is similar to the sequence reported for other MIP family members (Fig. 1). There was one protein kinase A phosphorylation site at Ser-257 and three protein kinase C phosphorylation sites at Ser-34, Ser-151 and Ser-229; no possible *N*-glycosylation site was found in the AQP-x5 protein. The amino acid sequence contained the conserved NPA motifs found in all MIP family members as well as a cysteine immediately upstream of the second NPA motif, the position of which is similar to that found in other anuran AQPs previously cloned (Hasegawa et al., 2003; Tanii et al., 2002). *Xenopus* AQP-x5 showed the highest degree of amino acid sequence homology to *Xenopus* AQP (99%, AY151156), followed by a high degree of homology to *Bufo*-t3 (79%, AF020623), human AQP2 (66%) (Sasaki et al., 1994), human AQP5 (64%) (Lee et al., 1996), rat AQP2 (65%) (Fushimi et al., 1993), rat AQP5 (64%) (Raina et al., 1995), mouse AQP2 (67%) (Rai et al., 1997), and mouse AQP5 (63%) (Krane et al., 1999). In addition, *Xenopus* AQP-x5 showed sequence homology – albeit at a lower degree – to *Hyla* AQP-h2 (57%) (Hasegawa et al., 2003) and *Hyla* AQP-h3 (58%) (Tanii et al., 2002). This sequence of full-length cDNA has been deposited in DDBJ/EMBL/GenBank (accession no. AB250090).

Distribution of Xenopus AQP-x5 mRNA expression in various tissues

The distribution of *Xenopus* AQP-x5 mRNA expression in various tissues was investigated by means of RT–PCR using total RNA from these tissues. AQP-x5 mRNA was observed in the ventral and dorsal skins, brain, lungs, and testes (Fig. 2). The RT–PCR results were confirmed by Southern blot analysis (data not shown).

Expression of Xenopus AQP-x5 in Xenopus oocytes

Transmembrane water flow through the *Xenopus* AQP-x5 was evaluated by determining the expression of the AQP in *Xenopus* oocytes. The oocytes were incubated for 3 days at 18°C, then transferred from isotonic (200 mOsm) to hypo-osmotic (70 mOsm) Barth's solution; the subsequent swelling was monitored under the microscope and recorded on an

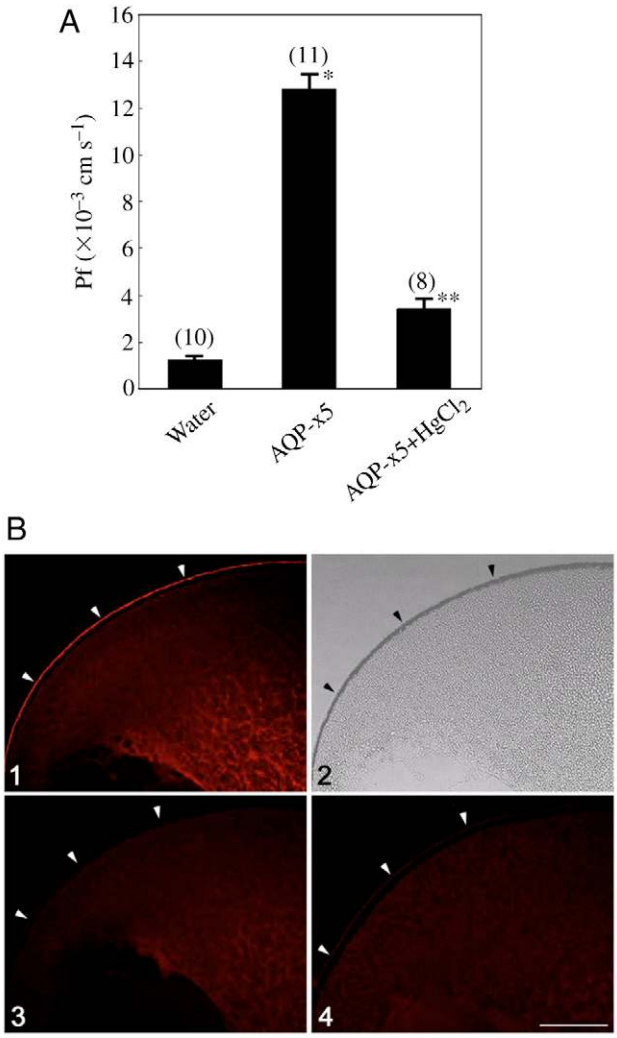

Fig. 3. Expression of AQP-x5 in *Xenopus* oocytes. (A) Osmotic water permeability (Pf) was calculated from the initial rate of oocyte swelling. Oocytes were microinjected with water or cRNA encoding AQP-x5. A portion of the AQP-x5-injected oocytes was incubated with 0.3 mmol l^{-1} $HgCl_2$. All data shown are given as the mean ± s.e.m. of measurements from 8–11 oocytes (as indicated above the bars) in each experimental group. *P<0.001 *vs* water, **P<0.001 *vs* AQP-x5. (B) Immunofluorescence images of AQP-x5 protein in AQP-x5-injected oocytes. (1) After completion of the swelling experiments, immunoreactive AQP-x5 substances are visible, predominantly in the plasma membrane. (2) The corresponding Nomarski differential interference image. (3) In the absorption test, positive immunoreactive substances obtained with anti-AQP-x5 are nearly eliminated at background levels in AQP-x5-injected oocyte. (4) As in 3, only background levels are seen in the water-injected oocyte with anti-AQP-x5. Arrowheads indicate plasma membrane. Scale bar, 50 μm.

attached CCD camera. The coefficient of osmotic water permeability (Pf) was then calculated (Fig. 3A). The AQP-x5 cRNA-injected oocytes showed significantly more swelling than the water-injected ones. The water permeability was completely inhibited by 0.3 mmol l^{-1} $HgCl_2$. When sections of the AQP-x5 cRNA-injected oocytes were immunostained with anti-*Xenopus* AQP-x5, a large immunofluorescent mass was seen in the plasma membrane (Fig. 3B1,2). The immunopositive sites were completely eliminated by preabsorption of the antiserum with 10 μg ml^{-1} of the immunogen peptide (Fig. 3B3). No indication of an immunolabel was found in the water-injected oocytes (Fig. 3B4).

Antibody specificity

The specificity of the anti-*Xenopus* AQP-x5 was tested using extracts of the AQP-x5 cRNA-injected oocytes. The antiserum detected a major band at 29.0 kDa in the extract (Fig. 4A). This immunopositive band was not detected when anti-AQP-x5 was preabsorbed with the peptide used as immunogen (Fig. 4B), effectively showing that this immunoreactive band was specific to the antiserum.

Localization of Xenopus AQP-x5 protein in the skin

Skin glands visualized in *Xenopus* skin sections treated by Mallory's triple staining procedure were easily recognizable as the granular gland, the mucous gland, and the small granular gland (Fig. 5). The small granular glands were located close to the epidermal layer of the skin and contained orange G-positive materials in their cytoplasm. When ventral or dorsal skin sections were stained with anti-AQP-x5, labels for AQP-x5 were predominately localized in the small granular gland, but not in the granular gland (Fig. 6A,B). In the small granular gland, the labels were found in the apical plasma membrane of the secretory cells (Fig. 6A). Weak labels for AQP-x5 were occasionally observed in the apical plasma membrane of the secretory cells located in the upper region of mucous gland (Fig. 6A,B). No signal for AQP-x5 was found in excretory duct cells and intercalated cells in the small granular and mucous glands (Fig. 6A,B). To confirm the specificity of the staining, we carried out a control experiment. Immunolabels for AQP-x5 in the secretory cells were eliminated when the antiserum was preincubated with the C-terminal peptide of the AQP-x5 protein used as an immunogen (Fig. 6C). When the sections were double-stained with the anti-V-ATPase E-subunit and AQP-x5, a positive reaction for V-ATPase was found in only a few of the secretory cells of the small granular gland (Fig. 6D). In addition, no positive signal for AQP-x5 was detected in the brain, lungs and testes (data not shown).

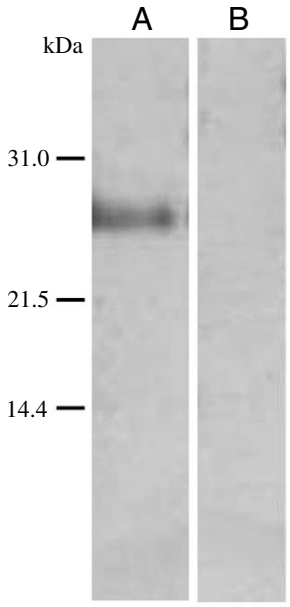

Fig. 4. Characterization of anti-AQP-x5 serum by western blot analysis. (A) Immunoreactive bands are seen at 29.0 kDa in an extract of AQP-x5 cRNA-injected oocytes. (B) The membrane was immunostained with the antiserum preabsorbed with the antigen peptide (10 μg ml^{-1}).

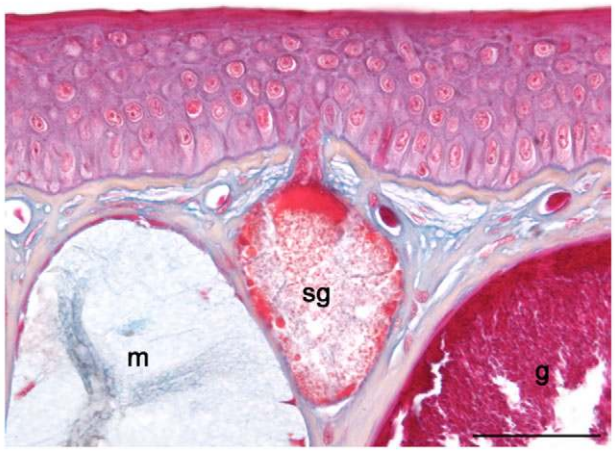

Fig. 5. Mallory's triple staining of *Xenopus* skin. In sections treated with Mallory's triple stain the skin glands can be classified into three types: the granular gland (g), the mucous gland (m), and the small granular gland (sg). Scale bar, 50 μm.

Expression and localization of other anuran amphibians

When skins of *Rana japonica*, *Hyla japonica* and *Bufo marinus* were immunostained with anti-AQP-x5, a positive reaction for AQP-x5 was seen in the apical plasma membrane of the mucous or seromucous glands in these anuran skins. A V-ATPase E-subunit-positive reaction was also found in specific cells in the glands, but these cells were different from the AQP-x5-positive ones (Fig. 7). However, these antisera did not stain any cells of the skins of *R. catesbeiana* and *R. nigromaculata* (data not shown).

Subcellular localization of Xenopus AQP-x5

Electron microscopy revealed that the two types of epithelial cells that are normally present in the small granular glands: one is characterized by having secretory granules and the other by

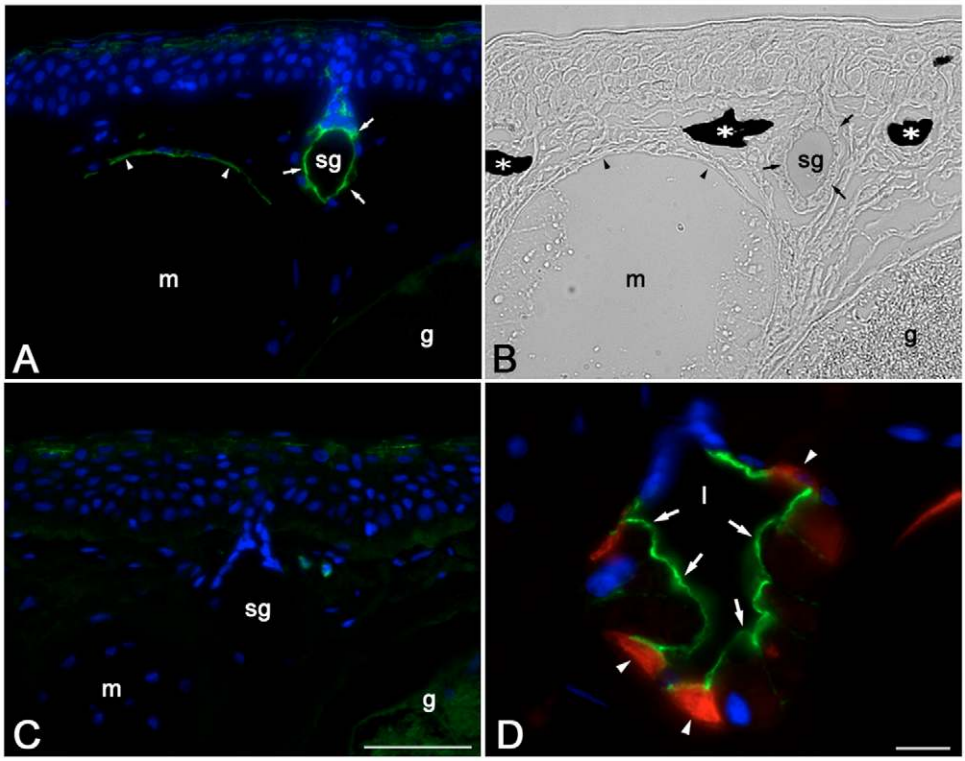

Fig. 6. Immunofluorescence localization of AQP-x5 in the skin glands. (A,C,D) Fluorescence images of AQP-x5 and (B) the corresponding Nomarski differential interference contrast image. (A,B) The labels (green; arrows) are clearly visible in the apical plasma membrane of granular cells in the small granular glands (sg). Weak positive reaction is visible in the apical plasma membrane of glandular cells located in upper sides of the mucous glands (arrowheads). No labeling is seen in the mucous (m) and granular (g) glands. The asterisk in B indicates red blood cells, displaying a nonspecific label for AQP-x5. (C) No labeling is detected in any of the cells of the skins when anti-AQP-x5 is preabsorbed with the corresponding antigen peptide. (D) Double-labeling for AQP-x5 (green) and V-ATPase E-subunit (red); V-ATPase-expressing cells are observed among the glandular cells (arrowheads). Nuclei are counterstained with DAPI (blue); l, lumen. Scale bars, 50 μm (A-C); 10 μm (D).

having numerous mitochondria (Fig. 8). These latter MR cells also have microvilli-like structures in the plasma membrane. When the ultrathin sections were immunolabeled for *Xenopus* AQP-x5, the labels were seen to be localized in the apical plasma membrane of the secretory cells (Fig. 9A). These labels for AQP-x5 were eliminated to background levels when the antiserum was preincubated with the C-terminal peptide of AQP-x5 protein used as an immunogen (Fig. 9B).

Discussion

We report here the full sequence of an mRNA encoding an AQP that is specifically expressed by the small granular glands found in the skin of *Xenopus*. This AQP – denoted AQP-x5 – was structurally characterized as having two NPA motifs and six putative transmembrane domains as well as a cysteine at a mercurial sensitivity site just upstream of the second NPA motif. A homology analysis revealed that the deduced amino acid sequence of *Xenopus* AQP-x5 has a high degree of homology to mammalian AQP5. Furthermore, AQP-x5 has a putative phosphorylation site for protein kinase A at Ser-257, which is identical to that of mammalian AQP2 (Fushimi et al., 1993) and AQP5 (Raina et al., 1995), and to *Hyla* AQP-h2 and AQP-h3 (Hasegawa et al., 2003; Tanii et al., 2002). Taken as a whole, these data suggest the possibility that water permeation in the small granular glands is regulated by a translocation mechanism similar to that observed for mammalian AQP2 in the kidney collecting duct cells, and for *Hyla* AQP-h2 and AQP-h3 in the granular cells of the tree frog urinary bladder and ventral pelvic skins, respectively.

Water-permeability experiments using *Xenopus* oocytes showed that AQP-x5 facilitates water permeation and that $HgCl_2$ inhibits it, suggesting that *Xenopus* AQP-x5 could be classified as a member of the AQP family.

Western blot analysis of AQP-x5 cRNA-injected oocytes showed one band at 29.0 kDa. In general, a distinct band is detected at the higher molecular mass, in a glycosylated form, but in the present investigation this band appeared to be absent. This result supports our other results in indicating that *N*-glycosylated sites are absent from the protein of *Xenopus* AQP-x5. Western blot analysis using the extract of *Xenopus* skins did not produce a positive reaction (data not shown), which may have be due to *Xenopus* skins containing only a very small amount of AQP-x5 protein. The western blot analysis carried out in the present investigation in which the preabsorbed antiserum was used also confirmed that this immunoreactive band is specific to the antiserum.

Amphibian skin glands have been classified into two major types: granular (serous or poison) and mucous glands. Mills and Prum (Mills and Prum, 1984) further subdivided the mucous glands of *Rana pipiens*, *R. temporaria* and *R. catesbeiana* as being either mucous or seromucous glands, whereas Fujikura et al. (Fujikura et al., 1988) distinguished between the small granular gland and the granular and mucous glands. However, MR cells interspersed with secretory cells are commonly present only in *Xenopus* small granular glands (Fujikura et al., 1988) and in the mucous glands of *R. pipiens*, *R. temporaria* and *R. catesbeiana* (Mills and Prum, 1984). Furthermore, the secretory activity of the serous glands is stimulated in response to isoproterenol, which is a β-adrenergic

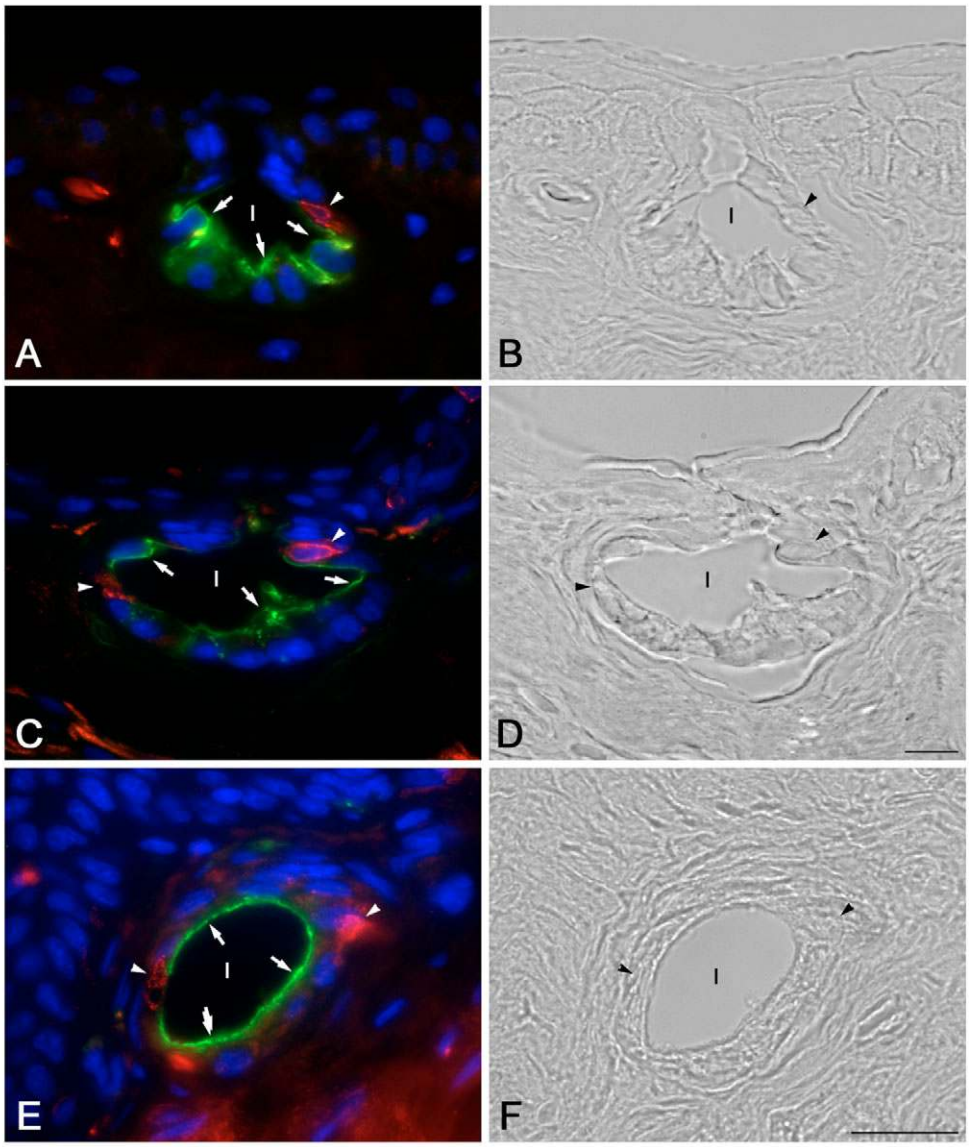

Fig. 7. Localization of AQP-x5 by immunofluorescent staining in *Hyla japonica* (A,B), *Rana japonica* (C,D) and *Bufo marinus* (E,F). Fluorescence images (A,C,E) for AQP-x5 and the corresponding Nomarski differential interference contrast images (B,D,F) are shown. AQP-x5 (green; arrows); V-ATPase E-subunit (red; arrowheads) are clearly visible. l, lumen. Scale bars, 10 μm (A–D); 50 μm (E,F).

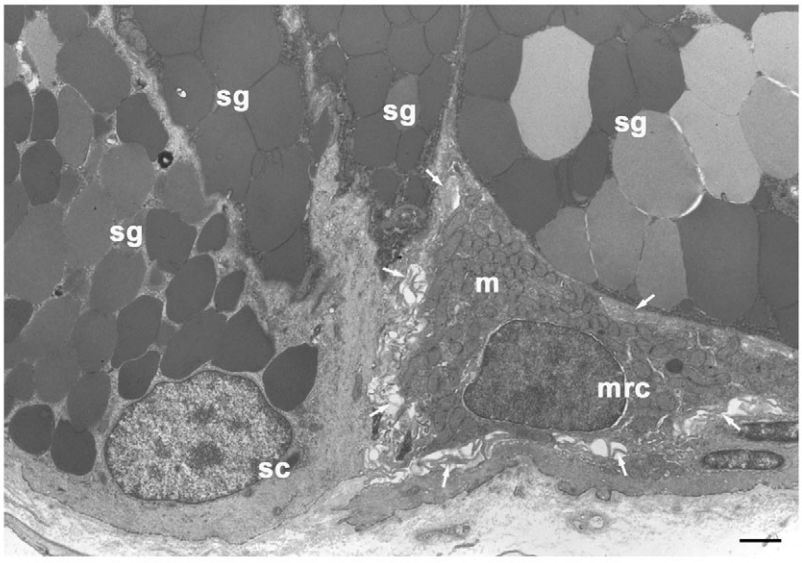

Fig. 8. A conventional electron micrograph showing secretory cells (sc) and a mitochondria-rich cell (mrc) in the small granular gland. The secretory cell has many secretory granules (sg), whereas the cytoplasm in the mitochondria-rich cell is filled with mitochondria, and microvilli-like structures (arrows) develop throughout the region of the plasma membrane. Scale bar, 1 μm.

agonist, whereas that of the small granular glands and mucous glands is not (Fujikura et al., 1988). Thus, the organization and physiological properties of amphibian skin glands may vary depending upon the species.

The immunofluorescence and immunoelectron microscopic investigations revealed that AQP-x5 was specifically expressed in the apical plasma membrane of the secretory cells in the small granular and mucous glands of the skins. This positive labeling pattern was somewhat different from that revealed in the RT–PCR experiments on the distribution of mRNA in the tissues. In the RT–PCR analyses, AQP-x5 mRNA was detected in the brain, lungs and testes, which is contrary to the immunocytochemistry results that showed no immunopositive cells in these tissues. We therefore assume that AQP-x5 in the brain, lungs and testes is translated at indiscernible levels, if at all. Comparative studies have demonstrated that AQP-x5 protein is located in the apical plasma membrane of the mucous or seromucous glands in several anuran species, suggesting that the AQP-x5 protein plays a pivotal role in water fluxes in the skin glands of anuran amphibians. Lillywhite (Lillywhite, 1971) demonstrated that discharge from the mucous glands in the skin of *Rana catesbeiana* increases as the body temperature rises, whereas dehydration does not affect glandular activity. The rise of body temperature during basking induces a slow and continuous release of mucus. This serves to maintain a moist integument and a steady state of evaporation water loss. Consequently, our findings indicating the presence of AQP-x5 protein in the apical plasma membrane of several skin glands suggests that the excretion of water from these skin glands is involved in the regulation of body temperature. The clawed toad *Xenopus* always lives in the water, but when the pools dry up in the summer it burrows into the mud to avoid desiccation, where it remains quiescent (aestivating) until the next rainy season. One possibility is that the small granular glands of *Xenopus* have developed as an adaptive mechanism for facilitating such a change of habitation.

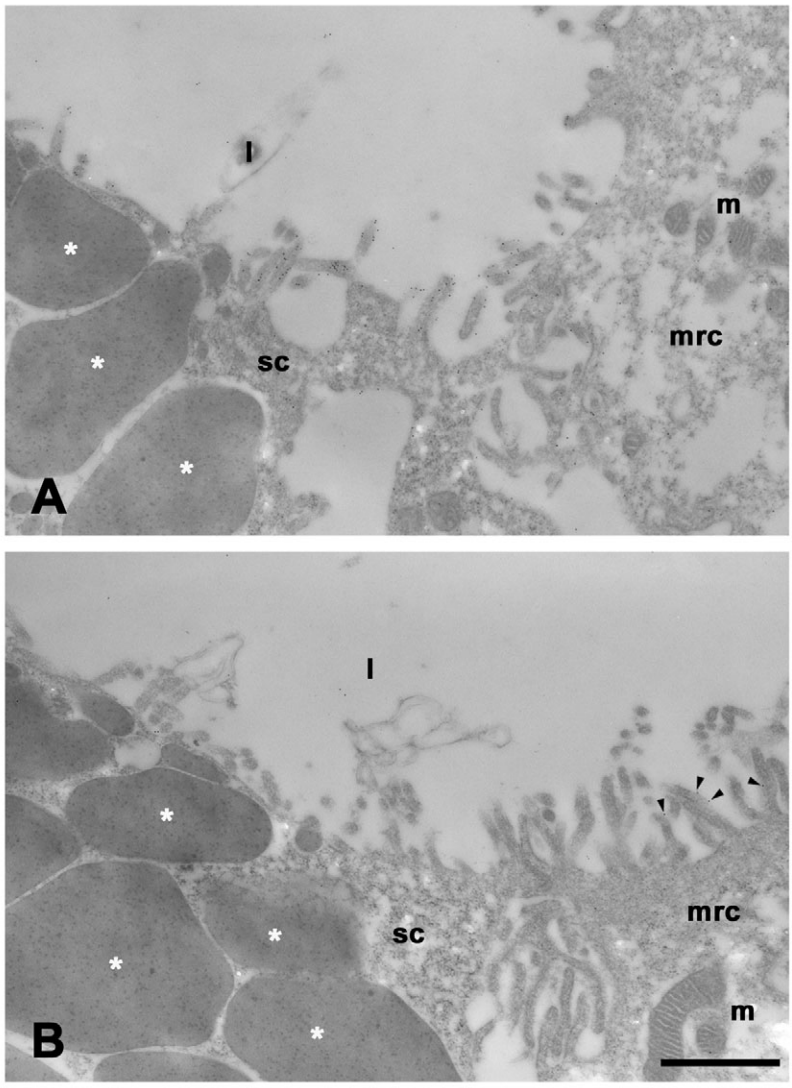

Fig. 9. (A) An immunoelectron micrograph showing the small granular gland immunolabeled for AQP-x5. Labels are visible in the apical plasma membrane of the secretory cells (sc) in the small granular gland. (B) An immunoelectron micrograph showing secretory and mitochondria-rich cells labeled with the antibody preabsorbed with the antigen. Immunogold particles were seen at background levels, especially in mitochondria-rich cell (arrowheads). l, lumen; mrc, mitochondria-rich cell; m, mitochondria; asterisks, secretory granule. Scale bar, 1 μm.

The small granular gland is characterized by having MR cells among the secretory cells (Fujikura et al., 1988). Electron microscopy revealed that the V-ATPase E-subunit-positive cell shows the properties of an MR cell. In amphibians, MR cells are known to be present in the skin (Katz et al., 2000; Willumsen et al., 2002; Whitear, 1972), urinary bladder (Choi, 1963; Wade, 1976), kidneys (Uchiyama and Yoshizawa, 2002) and endolymphatic sac (Yajima et al., 2005). Because MR cells contain carbonic anhydrase and V-ATPase (Brown and Breton, 1996; Wieczorek et al., 1999), these cells are considered to be involved in the export of protons. In our preliminary experiments, we examined the *Xenopus* small granular glands using electron microscopy after incubating the skins with bafilomycin A_1, a specific inhibitor of V-ATPase. However, we did not obtain any evidence that functional inhibition of V-ATPase affects the distribution and expression of AQP-x5. Further studies are required to define a functional relationship between MR cells and the presence of AQP-x5 in secretory cells of the small granular glands.

In summary, the present study provides further evidence of the physiological and ecological roles of amphibian skin glands in water adaptation.

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