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Salinity regulates claudin mRNA and protein expression in the teleost gill

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Tipsmark CK, Baltzegar DA, Ozden O, Grubb BJ, Borski RJ. Salinity regulates claudin mRNA and protein expression in the teleost gill. *Am J Physiol Regul Integr Comp Physiol* 294: R1004–R1014, 2008. First published January 9, 2008; doi:10.1152/ajpregu.00112.2007.—The teleost gill carries out NaCl uptake in freshwater (FW) and NaCl excretion in seawater (SW). This transformation with salinity requires close regulation of ion transporter capacity and epithelial permeability. This study investigates the regulation of tight-junctional claudins during salinity acclimation in fish. We identified claudin 3- and claudin 4-like immunoreactive proteins and examined their expression and that of select ion transporters by performing Western blot in tilapia (*Oreochromis mossambicus*) gill during FW and SW acclimation. Transfer of FW tilapia to SW increased plasma osmolality, which was corrected after 4 days, coinciding with increased gill Na^+-K^+-ATPase and Na^+-K^+-$2Cl^-$ cotransporter expression. Gill claudin 3- and claudin 4-like proteins were reduced with exposure to SW. Transfer to FW increased both claudin-like proteins. Immunohistochemistry shows that claudin 3-like protein was localized deep in the FW gill filament, whereas staining was found apically in SW gill. Claudin 4-like proteins are localized predominantly in the filament outer epithelial layer, and staining appears more intense in the gill of FW versus SW fish. In addition, tilapia claudin 28a and 30 genes were characterized, and mRNA expression was found to increase during FW acclimation. These studies are the first to detect putative claudin proteins in teleosts and show their localization and regulation with salinity in gill epithelium. The data indicate that claudins may be important in permeability changes associated with salinity acclimation and possibly the formation of deeper tight junctions in FW gill. This may reduce ion permeability, which is a critical facet of FW osmoregulation.

osmoregulation; tight junction; tilapia; epithelia

EURYHALINE FISH POSSESS the remarkable ability to compensate for disturbances in intracellular ion balance, cell volume, and protein function in the face of fluctuating ambient salinities (31, 37, 48). One of the key events in osmoregulatory corrections involves fine-tuning of the ion-transporting apparatus of the gill epithelia. This requires rearrangement of gill chloride cells and changes in gill Na^+-K^+-ATPase, Na^+-K^+-$2Cl^-$ cotransporter, and other critical ion transport proteins (12, 13). In most euryhaline teleosts, a strong upregulation of gill Na^+-K^+-ATPase (16, 31, 37) and Na^+-K^+-$2Cl^-$ cotransporter (32, 41, 47) is associated with seawater (SW) acclimation, whereas the opposite occurs during freshwater (FW) acclimation (41, 46).

In addition to ion transporter expression, the transcellular and intercellular permeability of the gill epithelium also contributes to overall epithelial transport characteristics. It has been known for a long time that the osmotic permeability of the eel gill decreases drastically during SW acclimation (8, 14). The biochemical basis for changes in water permeability may entail the robust downregulation of gill aquaporin mRNA and protein expression that accompanies SW acclimation in eel (6, 21). Although osmotic permeability decreases in SW, the conductance and short-circuit current is found to be larger in a SW teleost compared with a FW fish (8, 27). A simultaneous change in the ultrastructure of tight junctions (TJs) is also seen (28). Secretion of Na^+ is thought to involve a paracellular path confined to thin "leaky" TJs that occur in SW between mature chloride cells and accessory cells (19, 36), and this may contribute to the relatively high ionic permeability of the gill of marine compared with FW teleosts (17).

Claudins are four-transmembrane domain proteins that comprise a main component of TJ strands and are directly involved in its barrier function (38, 43). Claudin proteins of adjacent cell membranes interact with each other (10) to create charge- and size-selective pores in the paracellular space (1, 34, 35). Claudin 2 and 4, for instance, have been associated with paracellular cation permeability (2, 4). Acute regulation of claudins by phosphorylation may play a role in regulation of the paracellular barrier in mammals (11). Thus it has been demonstrated that claudin 3 and 4 are phosphorylated in ovarian cancer cells and that this results in increased paracellular permeability (7). Most data available, however, document hormone and cytokine regulation of claudin gene expression taking days to appear and in some cases accompanying paracellular permeability changes (44).

There are at least 24 claudins in the examined mammalian genomes (44) and 56 in the puffer fish, *Fugu rubripes* (23). In *Fugu*, gene duplication has apparently produced 17 genes closely related to mammalian claudin 3 and 4 (23). Homologs for some of these class V and VI claudin genes are also present in the distantly related *Danio rerio* (18), suggesting that the duplication occurred early in the development of teleosts. Messenger RNA coding the *F. rubripes* claudin genes is expressed in a tissue- and developmental-specific manner (23). Recent studies in the European eel show claudin 27 mRNA to be upregulated in FW compared with SW fish (15). Despite the crucial importance of TJ proteins in determining the characteristics of epithelia and the presence of numerous claudin genes in teleosts, examination of claudin proteins are lacking in fish or any nontetrapod. Moreover, outside of claudin 27 in eel, nothing is known about the salinity-dependent expression of claudin mRNA in teleosts.

The aim of this study was to investigate the regulation of, and hence the possible role that might be played by gill claudins in the salinity acclimation process of the euryhaline Mozambique tilapia. We examined gill localization and expression of claudin 3-like and claudin 4-like proteins in con-

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junction with branchial Na^+-K^+-ATPase α-subunit and Na^+-K^+-$2Cl^-$ cotransporter expression, using a combination of immunohistochemistry and dual infrared fluorescence channel Western detection methods during the SW and FW acclimation process. In addition, we cloned the cDNA sequence of two tilapia claudin genes, claudin 28a and claudin 30, and examined their expression profiles during both FW and SW challenge to determine whether claudin mRNA might be regulated by salinity in a fashion similar to protein expression.

MATERIALS AND METHODS

Animals

Two separate series of salinity challenges were performed. For gill protein analyses, male tilapia (*Oreochromis mossambicus*; 50–100 g) were maintained at a constant temperature (21–22°C) and photoperiod (12:12-h light-dark) in either FW (hardness, 125 mg/l; alkalinity, 200 mg/l; pH 8.2–8.3) or full-strength SW [32 parts per thousand (ppt); pH 8.2–8.5] for a minimum of 3 wk before experiments. Fish were fed 2% body weight daily with a pelleted feed (Southern States, Richmond, VA) except for 24 h before sampling during the salinity challenge experiments.

In a second salinity challenge to measure gill gene expression, male tilapia (50–100 g) were maintained at a constant temperature (24–25°C) and photoperiod (12:12-h light-dark) in either FW (hardness, 110 mg/l; alkalinity, 150 mg/l; pH 8.0) or two-thirds SW (22.5–25.0 ppt; pH 8.5) with feeding regimes identical to those of the first salinity challenge.

All experimental procedures were performed in accordance with the National Institutes of Health *Guide for the Care and Use of Laboratory Animals* and were approved by the North Carolina State University Animal Care and Use Committee.

Sampling

Tilapia were transferred directly from FW to SW and from SW to FW and were sampled at 0, 24, 96, and 168 h after transfer. Fish sham-transferred to FW or SW were used as controls. A similar salinity challenge scheme was used in the second salinity challenge, except that two-thirds SW was used instead of full-strength SW.

Fish were anesthetized in buffered MS-222. Blood was drawn from the caudal blood vessels into a heparinized syringe and kept on ice, and plasma was isolated by centrifugation. Before further sampling, the fish were killed by spinal sectioning and pitting of the brain. For Western blot analyses, one second gill arch was sampled from each fish, placed in chilled SEI buffer (in mM: 300 sucrose, 20 Na_2-EDTA, and 50 imidazole, pH 7.3), quickly frozen in liquid nitrogen, and stored at −80°C. A piece (~0.5 g) of muscle was sampled from the caudal region for determination of water content.

In the second salinity challenge, plasma was collected as described above for measures of plasma osmolality. A 100-mg piece of the second gill arch was removed, dissected from the supporting cartilage, and stored in chilled 1-ml aliquots of RNAlater (Ambion, Austin, TX). Tissue sections were incubated overnight at 4°C before RNA isolation.

Analyses

Plasma osmolality and muscle water content. Plasma osmolality (mosmol/kg) was measured by vapor pressure osmometry (Wescor, Logan, UT), and muscle water content was measured gravimetrically after drying at 90°C for 24 h.

Western blot analysis. Gill tissue was homogenized in chilled SEI buffer (in mM: 300 sucrose, 20 Na_2-EDTA, and 50 imidazole, pH 7.3) with protease inhibitor cocktail (P8340; Sigma Aldrich, St Louis, MO) using a hand-held glass homogenizer. To remove intact cells and larger cell fragments, the homogenate was centrifuged at 1,000 *g* for 20 min (4°C). A crude membrane fraction was isolated from the supernatant by a second centrifugation at 50,000 *g* for 30 min (4°C). The obtained pellet was resuspended, and protein content was measured using a microassay-based BCA protein assay (Pierce, Rockford, IL). Proteins were resolved by SDS-polyacrylamide gel electrophoresis. Sample buffer and sample reducing agent [both NuPAGE; Invitrogen, San Diego, CA; final concentration in the loaded samples (in mM): 141 Tris base, 106 Tris·HCl, 73 lithium dodecyl sulfate, 0.5 EDTA, and 50 1,4-dithiothretiol, plus 8% glycerol (vol/vol), 0.019% serva blue G250 (wt/vol), and 0.006% phenol red (wt/vol)] were added before heating at 80°C for 10 min. An equal quantity of membrane protein (10 μg) was loaded in all lanes. Coomassie staining confirmed that lanes were loaded with similar protein levels. Proteins were separated by gel electrophoresis using 4–12% bis-Tris gels (NuPAGE system; Invitrogen), and MES/SDS buffer (in mM: 50 MES, 50 Tris, 3.5 SDS, and 1 Na_2-EDTA, with the addition of NuPAGE antioxidant) at 200 V (XCell II SureLock; Invitrogen). Molecular size was estimated by including a prestained marker (Bio-Rad, Hercules, CA).

After electrophoresis, the gel was soaked for 30 min in transfer buffer (in mM: 25 Tris, 192 glycine, and 10% methanol) and immunoblotted onto nitrocellulose membranes (0.45 μm; Invitrogen) by submerged blotting for 1 h at 30 V (XCell II; Invitrogen). Membranes were blocked in Tris-buffered saline with Tween 20 (TBS-T) with LI-COR (Lincoln, NE) blocking buffer (1:1) and washed in TBS-T. Blots were incubated overnight (4°C) with Na^+-K^+-ATPase α-subunit (α5, 1:1,000; mouse IgG), Na^+-K^+-$2Cl^-$ cotransporter (T4, 1:1,000; mouse IgG), claudin 3 (Z23.JM; Invitrogen; 1:250; rabbit polyclonal), or claudin 4 antibodies (3E2C1; Invitrogen; 1:500; mouse IgG). After washing, membranes were incubated for 1 h with goat anti-mouse and/or goat anti-rabbit secondary antibodies conjugated to Alexa IRDye 680 or IRDye 800CW (LI-COR). Blotted proteins were detected and quantified using the Odyssey infrared imaging system (LI-COR). The claudin 3- and 4-like proteins were always visualized on the same blots at different wavelengths (claudin 3: rabbit polyclonal, 680 nm; claudin 4: mouse IgG, 800 nm; see e.g., Fig. 1). Band intensity values were expressed as a relative abundance to that observed in gills from fish at *time 0* of the experiments. The T4 and α5 antibodies were obtained from the Developmental Studies Hybridoma Bank developed under auspices of the National Institute of Child Health and Human Development and maintained by The University of Iowa (Department of Biological Sciences, Iowa City, IA). They react with conserved regions in the Na^+-K^+-ATPase α-subunit (α5; Ref. 39) and Na^+-K^+-$2Cl^-$ cotransporter (T4; Ref. 26) and have been used extensively in fish studies (see e.g., Refs. 22, 32, 41, 45, 47).

The polyclonal claudin 3 antibody and monoclonal claudin 4 antibody were raised against a 22-amino acid peptide sequence derived from the COOH-terminal region of mouse claudin 3 and human claudin 4, respectively. In the *F. rubripes* genome, the two epitopes show homology (42–100% over 4–20 amino acids) with the deduced amino acid sequence of several claudin genes (claudin 3 antibody: fugu claudin 1, 2, 3a, 3b, 3c, 5a, 14b, 27b, 30a, and 30c; claudin 4 antibody: fugu claudin 3a, 3c, 3d, 5a, 8b, 8c, 10b, 19, 20b, 23a, 23b, 27c, 27d, 28a, 28b, 28c, 29a, 29b, 30a, 30c, 30d, and 32a). Several of these are expressed at the mRNA level in the *F. rubripes* gill (fugu claudin 1, 3c, 5a, 8b, 14b, 27b, 28a, 28b, 28c 30a, 30c, and 30d; Ref. 23). The Atlantic salmon transcriptome is represented by >400,000 expressed sequence tags (ESTs) from a number of diverse tissues and libraries available at the National Center for Biotechnology Information. We have identified 26 salmon claudin isoforms using this resource, and 5 are found in gill libraries (data not shown). Four of these are homologous to fugu 27, 28a, 28b, and 30 (GenBank accession nos. CK877239, CK878575, DW180135, and DW180053, respectively). They all belong to class VI claudins that share strong similarity with mammalian claudins 3 and 4 (23). The presence of class VI claudins in the gill of euryhaline fish also has been confirmed in recent work on eel, where a claudin 27 homolog was found (15). It is therefore likely that the putative claudin 3- and claudin 4-like

proteins detected in this study belong to the class V/VI claudins, which are expressed in the gill of teleosts, but the particular fish claudin, whether it be the tilapia claudin 28a and 30 (see RESULTS) or some other class V/VI claudin, cannot be discerned.

Immunohistochemistry. Immunohistochemical detection of claudin-like proteins in gill was detected according to our previously established protocols used to detect a variety of ion transporter proteins and enzymes (42). Gill tissue (0.5 cm) were sampled and fixed in fresh, buffered paraformaldehyde (4%) for 4 h. After dehydration, tissues were embedded in paraffin and sectioned (6 μm). For antigen retrieval, slides were boiled in citrate buffer (10 mM sodium citrate and 0.05% Tween 20, pH 6.0) for 40 min at 95°C. Endogenous peroxidase activity was suppressed by treatment with hydrogen peroxide. Subsequently, blocking serum was applied for 1 h at room temperature (Vectastain kit; Vector Laboratories, Burlingame, CA). Sections were incubated with claudin 3 and claudin 4 antibodies at 1:200 dilutions in PBS (in mM: 137 NaCl, 3 KCl, 10 Na_2HPO_4, and 2 KH_2PO_4, pH 7.4) for 16 h at 4°C in a closed and moistened chamber. For negative controls, duplicate sections were treated with normal serum for the same time and temperature. In addition, the claudin antibody was preabsorbed overnight at 4°C with 200 molar excess of their respective blocking peptides to show the specificity of the immunostaining. Invitrogen kindly provided the blocking peptides. After 16 h, diluted biotinylated universal IgG (Vectastain kit; Vector Laboratories) was applied and slides were incubated for 60 min at room temperature. Vectastain ABC reagent was then added for 60 min at room temperature. To visualize the claudin proteins, we prepared 3,3′-diaminobenzidine (DAB; Sigma) according to the manufacturer's instructions, and it was applied to sections for 3 min. The DAB reaction was terminated by dipping the slides into water. Sections were counterstained with hematoxylin.

Gill RNA isolation and cDNA synthesis. Gill tissue total RNA was isolated by TRI reagent (Molecular Research Center, Cincinnati, OH) extraction with RNA quality confirmed by gel electrophoresis (1% agarose, 0.4 μg/ml ethidium bromide). RNA concentration and purity were determined by spectrophotometry using a Nanodrop ND-1000 before and after DNase treatment (DNA-free; Ambion). Absorbance values ($Abs_{260/280}$) in all samples were 1.8–2.0. One microgram of DNase-treated total RNA was used in a cDNA synthesis reaction using a High Capacity cDNA reverse transcription kit (Applied Biosystems, Foster City, CA).

Molecular cloning and sequencing. PCR reactions were performed using GoTaq polymerase and buffer with 10 mM dNTP mix and 10 μM primer concentrations (Promega, Madison, WI). Amplicons were cloned into pCR II TA cloning vector (Invitrogen) and transformed into JM109 series competent cells (Promega). Two selected clones from each amplicon were submitted to the University of Chicago Cancer Research Center for forward and reverse sequencing with universal primers.

Initial partial coding sequences were obtained by PCR with degenerate primers (Table 1). The claudin 28a primer pair was designed from a ClustalW multiple sequence alignment of *Fugu* claudin 3 homologs (claudin 3a–d; GenBank accession nos. AY55437, AY554378, AY554367, and AY554368, respectively) using Vector NTI sequence software (25). The claudin 30 primer pair was designed from a ClustalW pairwise alignment of ESTs *Lipochromis* sp. (DB871129) and *Salmo salar* (DW180053). The PCR cycling parameters for claudin 28a were as follows: 1 cycle at 95°C for 2 min; 35 cycles at 95°C for 30 s, 50°C for 30 s, and 72°C for 1 min; and 1 cycle of final extension at 72°C for 5 min. The claudin 30 amplification parameters were the same as for claudin 28a, with the annealing temperature reduced to 47°C.

Gene-specific primers (Table 1) were developed from the partial sequence clones for 5′ and 3′ rapid amplification of cDNA ends (RACE)-PCR using the FirstChoice RNA ligase-mediated RACE kit (Ambion), which promotes the capture of full 5′ sequence by enzymatic, 5′-monophosphate degradation of unprotected (methyl cap) mRNA template. Primer design, RNA treatment, and PCR amplification were performed in accordance with the manufacturer's protocol.

Contig sequence analysis was performed on all clones, using Vector NTI *Contig Express* software to generate full-length *O. mossambicus* claudin 28a and claudin 30 cDNA sequences. To verify the in silico contig analysis, we designed an additional primer pair (Table 1) for the full coding sequence of both genes, which spans all sequences in the contig alignment. The PCR cycling parameters for the claudin 28a coding sequence (CDS) were as follows: 1 cycle at 95°C for 2 min; 35 cycles at 95°C for 30 s, 57°C for 30 s, and 72°C for 75 s; and 1 cycle of final extension at 72°C for 5 min. The claudin 30 CDS amplification parameters were the same as for claudin 28a, except the annealing temperature was reduced to 55°C.

mRNA expression. Claudin 28a and 30 mRNA levels were measured using Syber green quantitative RT-PCR according to our pre-

Table 1. *Primer sequences used in the cloning of O. mossambicus claudin 28a and claudin 30 cDNA and gene expression analysis*

Gene	Primer Name	Primer Sequence (5′ to 3′)	Application
Cldn28a	Degenerate F	GCY YTG CCC ATG TGG A	Initial sequence (partial cds)
	Degenerate R	SGT GTK KGC TGA CCA	
	5R Primary	GCT CTG CAT GAC ACA GTT CAT CCA	5′ RACE-PCR
	5R Nested	CCG TCA CAA TGT TGG CTC CAA T	
	3R Primary	CTA TGA TTC TCT GCT GGC TCT TCC	3′ RACE-PCR
	3R Nested	TAT GGG AGT TAT CCT GGG CAT TGC	
	CDS F	ATG GTG TCA ATG GGA CGA CA	Full coding sequence
	CDS R	TCA AAC ATA GGC TCG GCT GG	
	QPCR F	GGT CAC AGC CTT CAT TGG AGC C	Gene expression (qRT-PCR)
	QPCR R	GAG CTC TGG CAG CCT GAA GA	
Cldn30	Degenerate F	CTG KGC ATY ATT GG	Initial sequence (partial cds)
	Degenerate R	CCW GCR ATG ATG AAG A	
	5R Primary	GGT CAT CCA GAT GCC TTC CC	5′ RACE-PCR
	5R Nested	GCC AGC AGG ATG CCA ATG ATA C	
	3R Primary	TAC ATG GGA AGG CAT CTG GA	3′ RACE-PCR
	3R Nested	GCT GCT CGA GCC CTT ACT AT	
	CDS F	ATG GTT TCA GCT GCT TTG CA	Full coding sequence
	CDS R	TCA AAC ATA GCC CTT GTT TG	
	QPCR F	GCA TTA TTG GCT GGA TTG GGG T	Gene expression (qRT-PCR)
	QPCR R	GCA TTG CAT CTG GCC TGT GCT	

Cldn28a, claudin 28a; Cldn30, claudin 30; F, forward primer; R, reverse primer; qPCR, quantitative polymerase chain reaction; RACE, rapid amplification of cDNA ends.

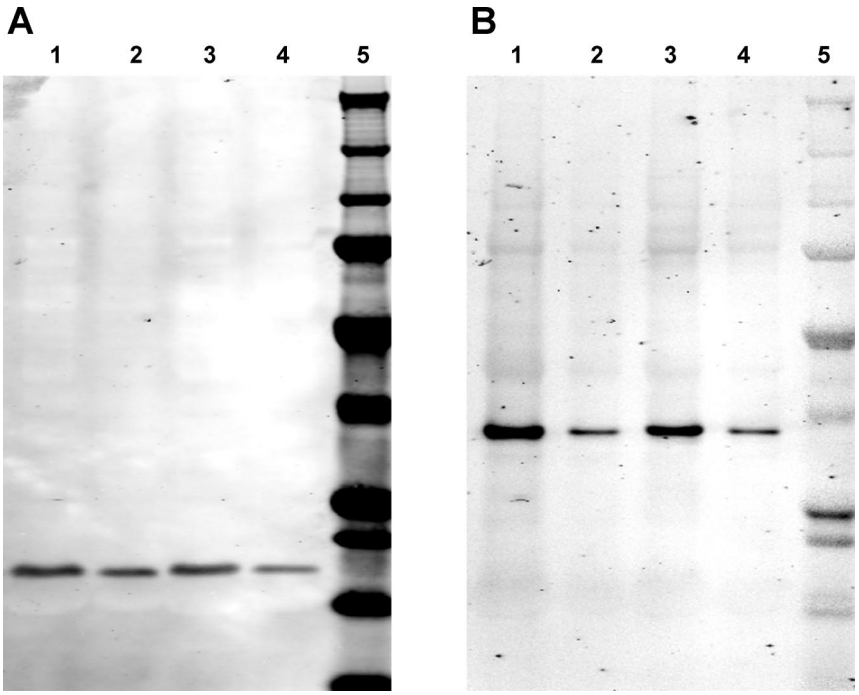

Fig. 1. Representative immunoblots of gill preparations recognizing claudin 3- and claudin 4-like proteins on the same blots at different wavelengths. *A*: freshwater (FW; *lanes 1* and *3*) and seawater preparations (SW; *lanes 2* and *4*) were probed with mouse claudin 3 antibody. *B*: FW (*lanes 1* and *3*) and SW preparations (*lanes* 2 and *4*) were probed with claudin 4 antibody. Molecular mass standards are displayed in *lane 5* in *A* and *B* (from *top* to *bottom*: 250, 150, 100, 75, 50, 37, 25, 20, 15, and 10 kDa).

viously established protocol (33). Gene-specific primers to the coding sequence of *O. mossambicus* claudin 28a and claudin 30 were developed using the Vector NTI sequence software package (Table 1). Real-time quantitative RT-PCR analysis was performed on a 20-ng total RNA sample with Brilliant Syber green master mix (Stratagene) containing 1.5 μM gene-specific primers on an ABI 7900 HT sequence detection system. After an initial denaturation at 95°C for 10 min, 40 cycles of PCR were performed, each consisting of denatur-

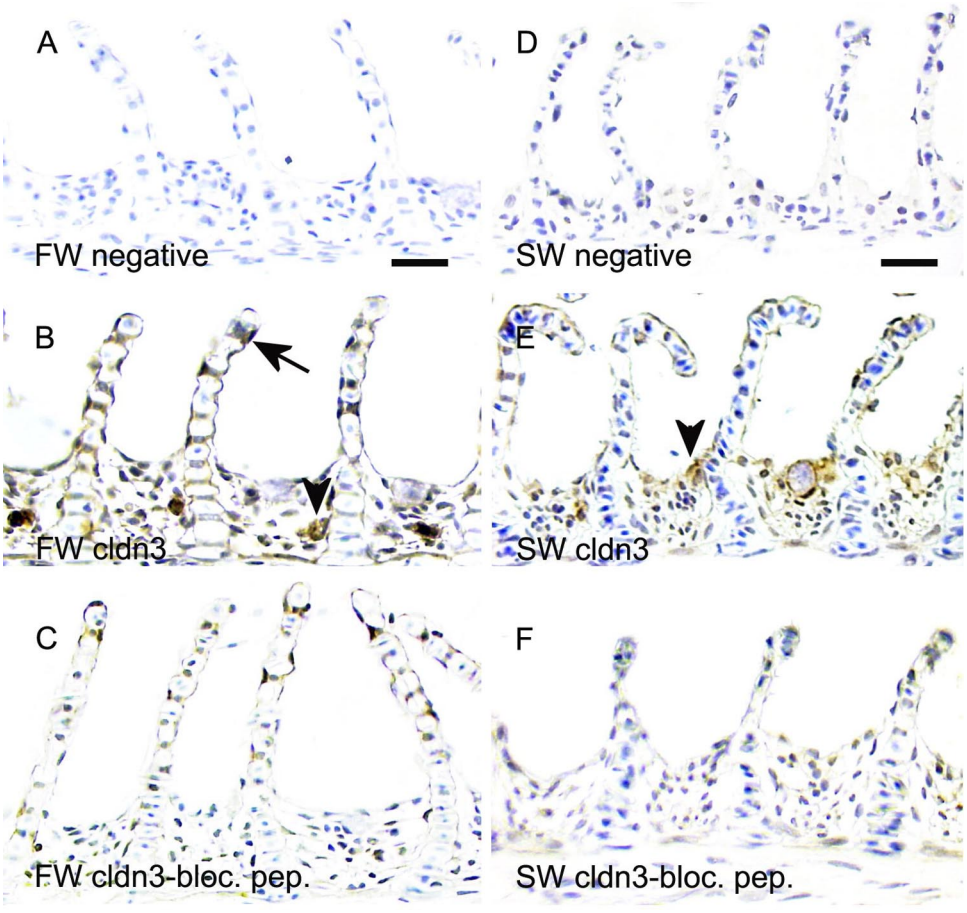

Fig. 2. Gill sections (6 μm) from FW (*A–C*) and SW tilapia (*D–F*), immunostained with the claudin 3 antibody (cldn3). Staining was specific, as verified by the lack of staining with normal serum (*A* and *D*) and when the antibody was preabsorbed with blocking peptide (cldn3-bloc. pep.; *C* and *F*). Sections were counterstained with hematoxylin. The arrowheads and arrow indicate examples of localized staining in the filament and on the lamellae, respectively. Bar, 20 μm.

ation at 95°C for 30 s, annealing at 60°C for 60 s, and extension at 72°C for 60 s. Real-time measurements were made during the extension step. Cycle threshold (C_t) values for experimental samples were transformed using a standard curve of serially diluted cDNA compared with C_t values (R^2 = 0.93–0.99). All mRNA data were normalized to total RNA concentration (4, 33) and are depicted as cDNA template per nanogram of total RNA. Primer specificity was assessed by melting curve analysis and, in addition, by visual inspection of sample amplicons using gel electrophoresis.

Statistics

A two-factorial ANOVA was used to analyze for overall effects of salinity change (FW or SW), time of sampling, and interaction between factors. The group of fish sampled at time 0 h was treated statistically as both a FW and SW group. When required, transformation of data was done to meet the ANOVA assumption of homogeneity of variances (assessed by residual plots). A priori, it was decided to compare FW and SW groups at the same time point. When the two-way ANOVA showed that the interaction between factors was significant, groups were compared with Bonferroni-adjusted Fisher's least significant difference test. All statistical analyses were performed using Statistica 7.0 (Tulsa, OK), and significant differences were accepted when $P < 0.05$.

RESULTS

On immunoblots of gill protein preparations, the antibody directed against mammalian claudin 3 revealed one single band with apparent molecular mass of 18 kDa (Fig. 1*A*). The claudin 4 antibody also detected only one protein band, this one having an apparent molecular mass of 34 kDa (Fig. 1*B*). These distinct bands were of the same size in the gill of both FW- and SW-acclimated tilapia. Gill claudin 3- and claudin 4-like protein expression was lower in long-term SW-acclimated (>3 wk) tilapia than in FW-acclimated fish (Fig. 1).

To examine the cellular site of claudin-like protein expression in the gill, we examined localization of claudin 3- and claudin 4-like proteins by immunostaining as shown in Figs. 2 and 3, respectively. In the gill of FW fish, claudin 3 staining was localized mainly in the deeper cell layers in the filament but also in pillar cells on the lamellae (Fig. 2*B*). In the SW gill, claudin 3 antibody staining was closer to the apical side of the filament (Fig. 2*E*). In the FW gill, the claudin 4 antibody stained epithelial cells in the filament but also some cells on the lamellae, including pillar cells (Fig. 3*B*). In the SW gill, claudin 4 antibody staining was substantially weaker, found mostly in a thin layer on the apical side of epithelial cells of the filament (Fig. 3*E*); no staining was seen in pillar cells (Fig. 3*E*). Negative staining was prepared by replacing primary antibody with normal serum, and this abolished the reaction (Figs. 2, *A* and *D*, and 3, *A* and *D*). Binding of both antibodies to gill cells was blocked when preabsorbed with their respective blocking peptides, which shows that staining was specific for both claudin antibodies (Figs. 2, *C* and *F*, and 3, *C* and *F*).

To further investigate the response of the putative gill claudins to salinity change, we performed time-course salinity transfer experiments. Transfer of FW-acclimated fish to SW induced an increase in plasma osmolality from ~330 to 380 mosmol/kg after 24 h (Fig. 4*A*). After 4 days, plasma osmolality was restored to levels typically observed in long-term SW-acclimated fish (350 mosmol/kg), as shown in Fig. 4*E*. Muscle water content decreased significantly after 24 h in response to SW but returned to control levels by 4 days (Fig. 4*B*). The expression of gill Na^+-K^+-ATPase α-subunit protein and Na^+-K^+-$2Cl^-$ cotransporter protein both increased 4 days after transfer to SW (Fig. 4, *C* and *D*). By contrast, the expression of claudin 3- and claudin 4-like proteins in gill

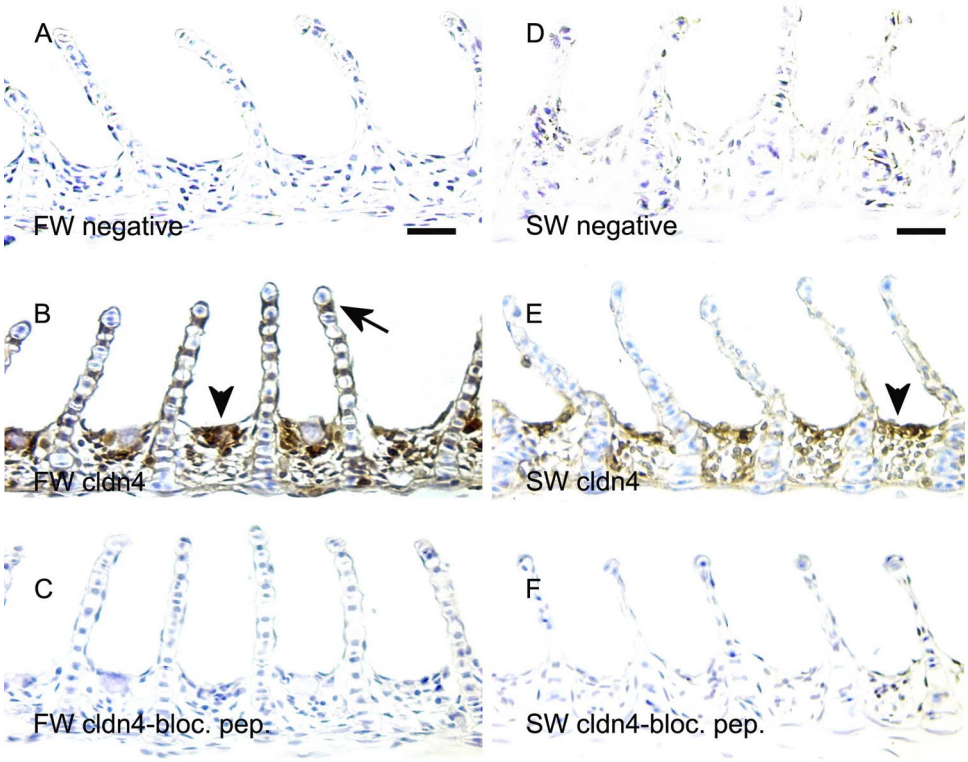

Fig. 3. Gill sections (6 μm) from FW (*A–C*) and SW tilapia (*D–F*), immunostained with the claudin 4 antibody (cldn4). Staining was specific, as verified by the lack of staining with normal serum (*A* and *D*) and when the antibody was preabsorbed with blocking peptide (cldn4-bloc. pep.; *C* and *F*). Sections were counterstained with hematoxylin. The arrowheads and arrow indicate examples of localized staining in the filament and on the lamellae, respectively. Bar, 20 μm.

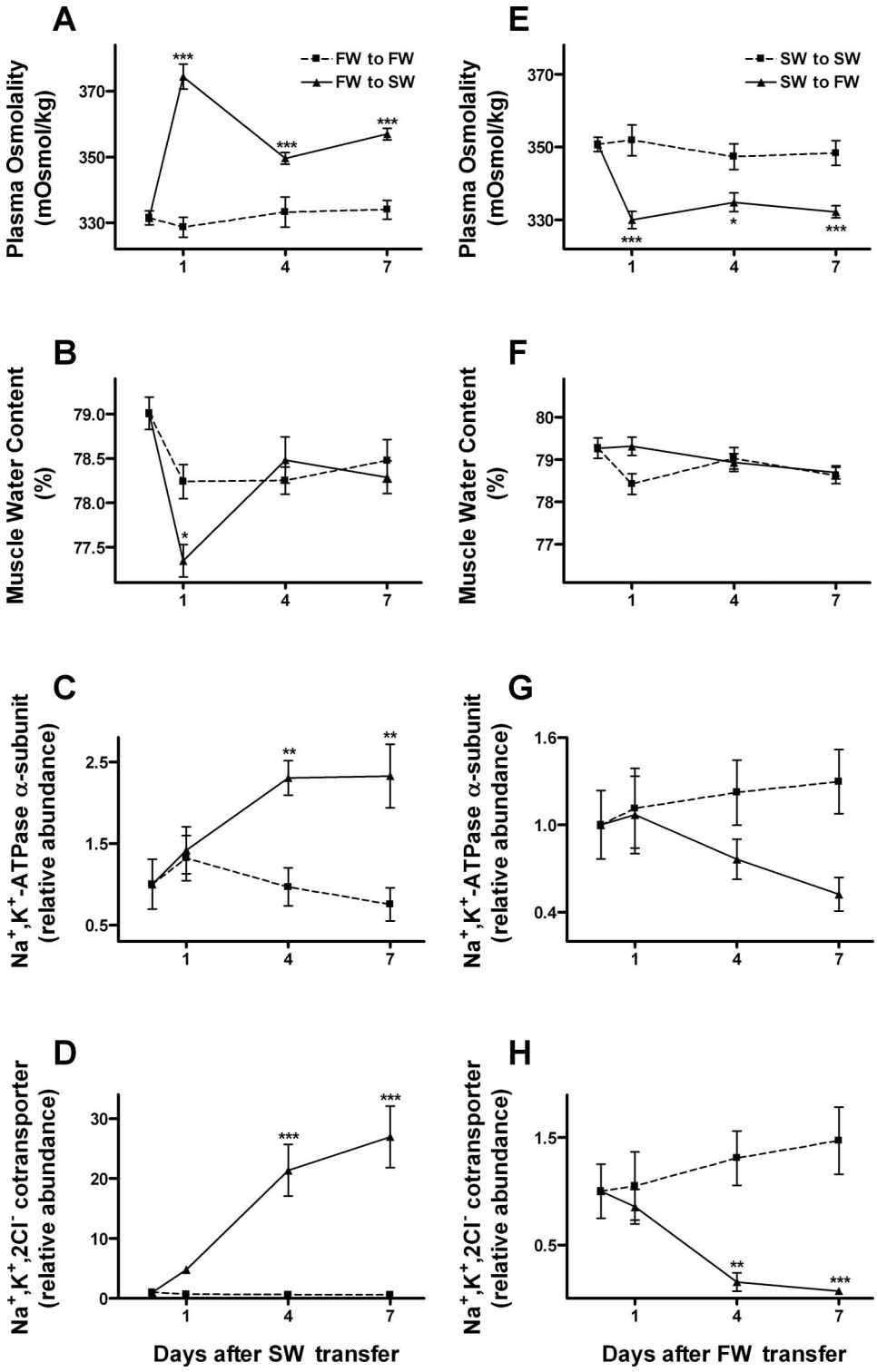

Fig. 4. Effect of salinity transfer on plasma osmolality, muscle water content, gill Na^+-K^+-ATPase α-subunit protein expression, and gill Na^+-K^+-$2Cl^-$ cotransporter protein expression. Data in *A–D* reflect fish transferred from FW to SW; data in *E–H* represent fish transferred from SW to FW at *time 0*: FW to FW, FW control fish sham-transferred to FW; SW to SW, SW control fish sham-transferred to SW. Overall, transfer to SW increased plasma osmolality (*A*; $P < 0.001$) and increased expression of Na^+-K^+-ATPase (*C*; $P < 0.01$) and Na^+-K^+-$2Cl^-$ cotransporter (*D*; $P < 0.001$). There was an overall effect of time of sampling for plasma osmolality, muscle water content, and Na^+-K^+-$2Cl^-$ cotransporter (*A*, *B*, and *D*; $P < 0.001$). There also was interaction between the 2 factors for plasma osmolality (*A*; $P < 0.001$), muscle water content (*B*; $P < 0.05$), Na^+-K^+-ATPase (*C*; $P < 0.05$), and Na^+-K^+-$2Cl^-$ cotransporter (*D*; $P < 0.001$). Transfer of SW fish to FW reduced plasma osmolality (*E*; $P < 0.001$) and expression of Na^+-K^+-ATPase (*G*; $P < 0.05$) and Na^+-K^+-$2Cl^-$ cotransporter (*H*; $P < 0.001$). There was an overall effect of time of sampling for plasma osmolality (*E*; $P < 0.001$) and a significant interaction between salinity and time for both plasma osmolality and Na^+-K^+-$2Cl^-$ cotransporter (*E* and *H*; $P < 0.01$). Values are means ± SE ($n = 10$, except for gill protein expression analysis, where $n = 5$). $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$, significant effects compared with sham controls.

declined in response to SW (Fig. 5, *A* and *B*; $P < 0.01$ for claudin 3 and $P < 0.05$ for claudin 4). For the claudin 3-like protein, there was a significant interaction between salinity and acclimation period, signifying the time dependence of the SW effect (significant after 7 days, $P < 0.01$; Fig. 5*A*).

Transfer of SW-acclimated fish to FW induced a rapid decrease in plasma osmolality from ~350 to 330 mosmol/kg after 24 h (Fig. 4*E*), and it settled at this level over the remainder of the experiment. No change in muscle water content was observed in this experiment (Fig. 4*F*). There was an overall significant reduction in the expression of gill Na^+-K^+-ATPase α-subunit protein ($P < 0.05$) and Na^+-K^+-$2Cl^-$ cotransporter protein ($P < 0.001$) in response to FW transfer (Fig. 4, *G* and *H*). For the Na^+-K^+-$2Cl^-$ cotransporter protein, there was a significant interaction between salinity and time, signifying that the protein declines with a greater significance

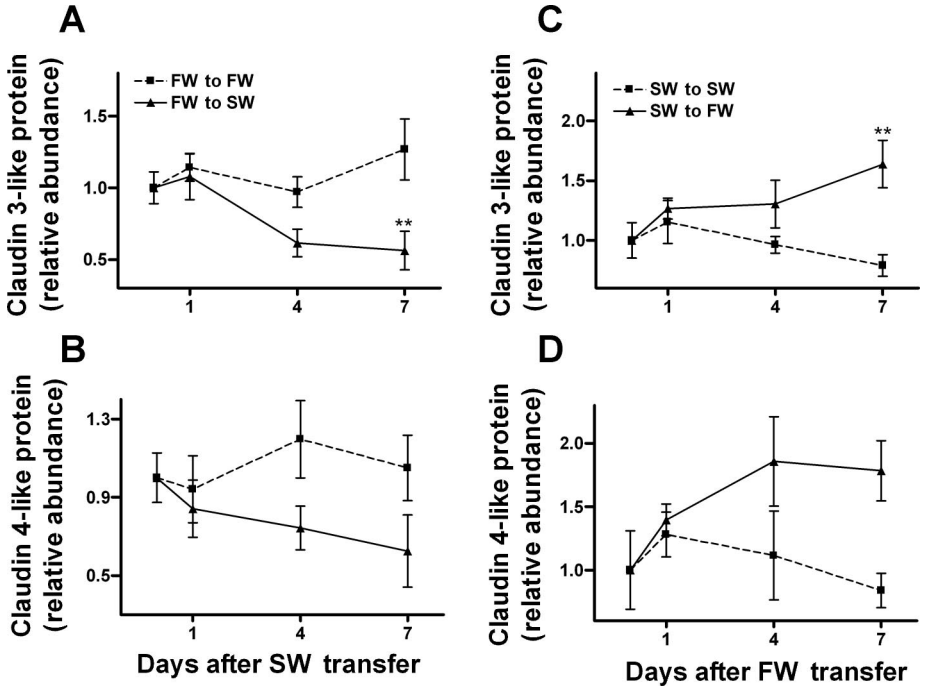

Fig. 5. Effect of salinity transfer on gill expression of claudin 3-like (*A* and *C*) and claudin 4-like proteins (*B* and *D*). Data in *A* and *B* reflect fish transferred from FW to SW; data in *C* and *D* represent fish transferred from SW to FW at *time 0*. Overall, transfer to SW decreased expression of claudin 3-like (*A*; $P < 0.01$) and claudin 4-like proteins (*B*; $P < 0.05$). There was no effect of time of sampling, but a significant interaction between the 2 factors was observed for the claudin 3-like protein (*A*; $P < 0.05$). Overall, FW transfer increased expression of claudin 3-like (*C*; $P < 0.01$) and claudin 4-like proteins (*D*; $P < 0.05$). There was no effect of time of sampling, but a significant interaction between time of sampling and salinity was observed for claudin-3 like protein expression (*C*; $P < 0.05$). Values are means ± SE ($n = 5$). **$P < 0.01$, significant effects compared with sham controls.

over the course of FW exposure (significant after 4 days; Fig. 4*H*). By contrast, the expression of claudin 3- and claudin 4-like proteins increased in the gill in response to FW (Fig. 5, *C* and *D*; $P < 0.01$ for claudin 3 and $P < 0.05$ for claudin 4). For the claudin 3-like protein, there was a significant interaction between salinity and time, being significant after 7 days of transfer to FW ($P < 0.01$; Fig. 5*C*).

Full-length cDNA sequences of tilapia (*O. mossambicus*) claudin 28a (GenBank accession no. EU344800) and claudin 30 (EU344801) were characterized by RACE-PCR of partial coding sequence clones. Analyses of claudin 28a indicated a single 1,175-bp cDNA sequence with 4× or greater coverage. The translated 633-bp open reading frame (ORF) shares the highest amino acid similarity (94%) with *Takifugu rubripes* claudin 28a and shares strong similarity to the class V/VI claudins (NCBI bl2seq; AAT64053; Refs. 23, 40). The deduced claudin 28a amino acid sequence shows 85 and 84% similarity to mouse claudin 3 (NP_034032) and human claudin 4 (NP_001296) proteins, respectively (Fig. 6). Tilapia claudin 30 analyses produced a 1,184-bp cDNA sequence with 4× coverage. A translated tilapia claudin 30 ORF (633 bp) shares equal similarity (85%) to the "a" and "c" paralogs of *T. rubripes* claudin 30 (claudin 30a, AAT64050; claudin 30c, AAT64059). Pairwise comparisons to mammalian claudins show 80% similarity to mouse claudin 3 and 83% similarity to human claudin 4 protein sequences (Fig. 6). An additional coding sequence PCR, spanning all segments of the gene, was performed to supplement the in silico contig analysis. The amplicons verified the predicted nucleotide sequence for both claudin 28a and claudin 30.

Tilapia claudin 28a and claudin 30 mRNA levels were determined by quantitative RT-PCR analyses in fish challenged with FW or two-thirds SW. As in the first salinity study, a sharp rise in plasma osmolality was observed 24 h after FW-acclimated fish were transferred to SW ($P < 0.001$; Fig. 7*A*). Osmolality was subsequently restored to baseline by 4 days. Claudin 28a and claudin 30 mRNA declined from initial *time 0* levels through *day 4* in both the SW and sham-transferred control groups (Fig. 7, *B* and *C*). By *day 7*, levels of both genes were lower in SW-transferred fish than control animals, although no statistically significant differences were observed.

Plasma solute concentration dropped sharply in SW-acclimated fish transferred to FW and remained lower than sham-transferred control fish at *days 1* and *4* ($P < 0.001$; Fig. 7*D*). Levels subsequently returned to levels observed in SW-acclimated fish by *day 7*. Claudin 28a mRNA levels increased by 45% in FW-transferred fish by 24 h, the earliest time point measured ($P < 0.05$; Fig. 7*E*). Levels of claudin 28a mRNA continued to increase in FW-transferred fish and remained elevated over sham-transferred control fish throughout the course of the experiment ($P < 0.001$; Fig. 7*E*). Claudin 30 mRNA levels were also significantly higher than the control group at *days 1* and *7* ($P < 0.05$; Fig. 7*F*) but not at *day 4* of transfer, where an elevated transcript level was observed in the control group.

DISCUSSION

Claudins are membrane proteins involved in the formation of TJs in epithelial tissue, and different isoforms confer distinctive charge and size selectivity of pores within these junctions (43). The relationship between protein expression of claudins in the gill and salinity acclimation of euryhaline fishes is unknown, although it is clear that major permeability changes occur in gill epithelia and operculum membranes during salinity acclimations (8, 31) and that genes for various forms of claudins are expressed in fish gills (3, 15, 23). In the present investigations we therefore assessed the dynamics of gill expression of claudin mRNA and proteins along with ion transporter proteins in tilapia during acclimation to either FW or SW.

A

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Mmus3     -MSMGLEITG TSLAVLGWLC TIVCCALPMW RVSAFIGSSI ITAQITWEGL  [ 50]
Omos28a   MV...RQML. FA..II.F.G ..II..M... K.T....AN. V....I....  [ 50]
Omos30    MV.AA.QML. .A.CII..IG V..I....Q. K.T....QN. V...T....I  [ 50]

Mmus3     WMNCVVQSTG QMQCKMYDSL LALPQDLQAA RALIVVSILL AAFGLLVALV  [100]
Omos28a   .....M.... .....I.... .......... ...V.IA.IV .FM.VILGIA  [100]
Omos30    ..T.......  .....V...M ...SS.....  ...TIIA..I GII.I.L.MA  [100]

Mmus3     GAQCTNCVQD ETAKAKITIV AGVLFLLAAL LTLVPVSWSA NTIIRDFYNP  [150]
Omos28a   .GK...F.EE QR..SRVA.A ...V.IC.GV .V.I..C...  ....QN....  [150]
Omos30    .GK.....E. .GS.S..G.A ...V.II.GV .C....C.T.  ....QE....  [150]

Mmus3     LVPEAQKREM GAGLYVGWAA AALQLLGGAL LCCSCPPRDK YAPTKILYSA  [200]
Omos28a   TLIN..R...  ..A..I..GT ...LI.....  ..S....KES PE---YPVKY  [200]
Omos30    MFNAS..K.L  ..S.FI..G. GG.LII....  ..AN...K.N .S---AK...  [200]

Mmus3     PRSTGPGTGT GTAYDRKDYV  [220]
Omos28a   GGARSVA.SR AYV-------  [220]
Omos30    ...SA.A.NK .YV-------  [220]
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B

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Hsap4     MASMGLQVMG IALAVLGWLA VMLCCALPMW RVTAFIGSNI VTSQTIWEGL  [ 50]
Omos28a   .V...R.ML. F...II.F.G TIII..M... K......A.. ..A.I.....  [ 50]
Omos30    .V.AA..ML. T..CII..IG .IVI....Q. K......Q.. ..A..T...I  [ 50]

Hsap4     WMNCVVQSTG QMQCKVYDSL LALPQDLQAA RALVIISIIV AALGVLLSVV  [100]
Omos28a   .....M.... .....I.... .......... ....V.A... .FM..I.GIA  [100]
Omos30    ..T.......  .........M ...SS.....  ...T..A.LI GII.I..AMA  [100]

Hsap4     GGKCTNCLED ESAKAKTMIV AGVVFLLAGL MVIVPVSWTA HNIIQDFYNP  [150]
Omos28a   ......FV.E QR..SRVA.A .....IC..V L.LI..C.S. NT...N....  [150]
Omos30    .......V..  .GS.S.IG.A .....II..V LCL...C... NT...E....  [150]

Hsap4     LVASGQKREM GASLYVGWAA SGLLLLGGGL LCCNCPPRTD KPYSAKYSAA  [200]
Omos28a   TLINA.R...  ..A..I..GT AA..I...A.  ..SS...KES PE.PV..GG.  [200]
Omos30    MFNAS..K.L  ....FI..G. G...II..A.  ..A....KDN YSAKYSAPRS  [200]

Hsap4     RSAAASNYV-  [210]
Omos28a   ..V.T.RAYV  [210]
Omos30    SAP.TNKGYV  [210]
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Fig. 6. ClustalW amino acid alignment of tilapia (*Oreochromis mossambicus*) claudin 28a (GenBank accession no. EU344800) and claudin 30 (EU344801) translated sequences to mouse (*Mus musculus*) claudin 3 (NP_034032; *A*) and human (*Homo sapiens*) claudin 4 proteins (NP_001296; *B*).

Transfer of FW-acclimated tilapia to SW induced an initial osmotic stress after 24 h. This was followed by acclimation to the new salinity within 4 days as judged by the restoration of muscle water content to pretransfer levels and plasma osmolality to levels typically found in fish fully acclimated to SW (~350 mosmol/kg; Fig. 4). The regain of osmotic balance coincides with major increases in cotransporter and Na^+-K^+-ATPase abundance (Fig. 4, *C* and *D*). This correlation of increased levels of branchial Na^+-K^+-$2Cl^-$ cotransporter and Na^+-K^+-ATPase and SW acclimation is in accord with earlier studies (24, 31, 41, 45, 47). Transfer of SW-acclimated fish to FW, on the other hand, induced a decrease in plasma osmolality to levels typically found in long-term FW-acclimated fish (~330 mosmol/kg) but no significant change in muscle water content (Fig. 4, *E* and *F*). This finding suggests that acclimation to FW may not induce as dramatic an initial osmoregulatory stress as is observed during SW acclimation, which is in agreement with that previously reported for this and other euryhaline species (brown trout, Ref. 42; Mozambique tilapia, Ref. 22). The decline in gill abundance of cotransporter and Na^+-K^+-ATPase following FW transfer (Fig. 4, *G* and *H*) may be linked to downregulation of unused hyposmoregulatory capacity, since these proteins are highly active in NaCl excretion and the SW acclimation process.

The number, size, and morphology of chloride cells and expression of gill transport proteins are dynamically regulated by both salinity and hormones (8, 30). In SW, transcellular extrusion of chloride and paracellular extrusion of sodium are dependent on chloride cell and transporter protein remodeling. To understand the paracellular pathway for Na^+ ions, a description of claudins and their regulation is essential but was, until now, lacking.

We used the Odyssey system with two infrared fluorescence channels for Western blot analysis that enabled simultaneous two-color target detection and, therefore, the simultaneous analyses of two putative claudins on the same blots. The employed mammalian claudin 3 and claudin 4 antibodies each detected one distinct gill protein band with an apparent molecular mass of 18 and 34 kDa, respectively. These apparent sizes are in the range that would be predicted for known teleost

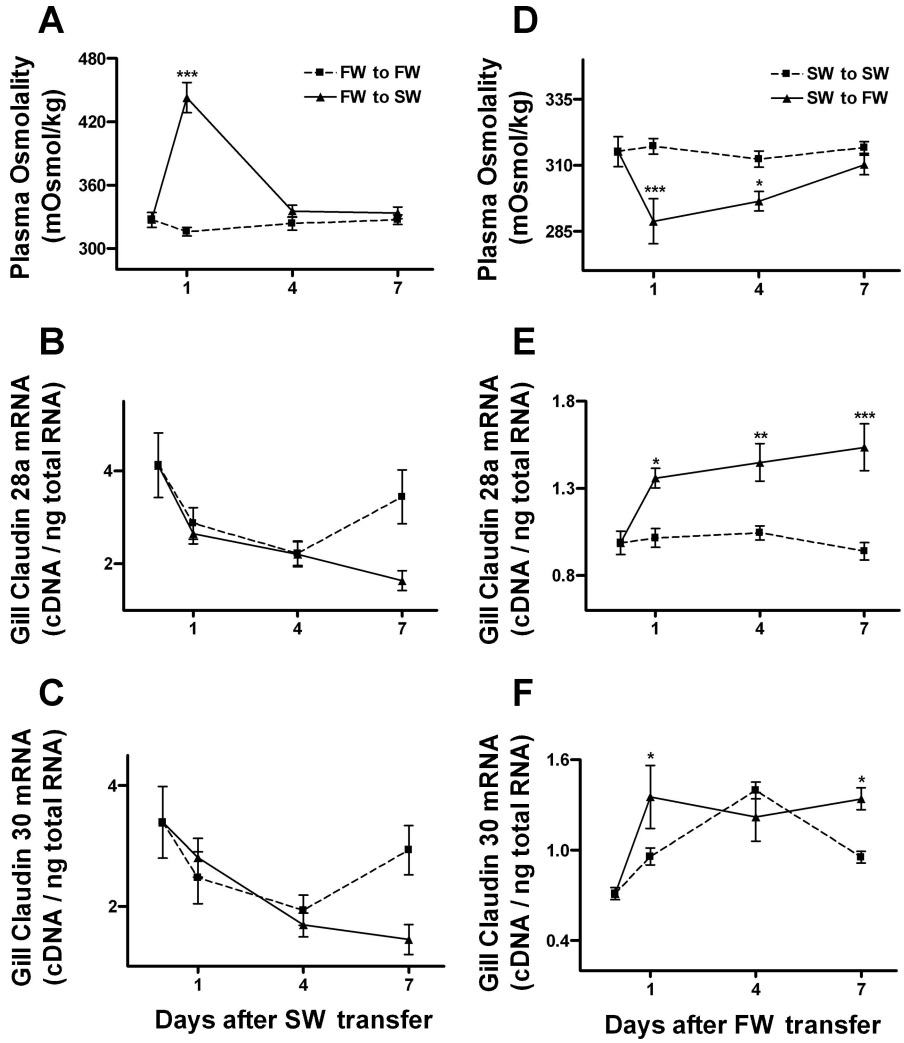

Fig. 7. Effects of salinity transfer on plasma osmolality and gill claudin 28a and claudin 30 mRNA levels. Data in *A–C* reflect fish transferred from FW to SW; data in *D–F* represent fish transferred from SW to FW at *time 0*. Overall, transfer to SW increased plasma osmolality (*A*; $P < 0.001$). There was an overall effect of time of sampling for plasma osmolality, claudin 28a mRNA, and claudin 30 mRNA (*A–C*, respectively; $P < 0.01$). There also was interaction between the 2 factors for plasma osmolality (*A*; $P < 0.001$). Transfer of SW fish to FW caused an overall reduction in plasma osmolality (*D*; $P < 0.001$) and increased expression of claudin 28a (*E*; $P < 0.001$) and claudin 30 mRNA (*F*; $P < 0.05$). There was an overall effect of time of sampling for plasma osmolality (*D*; $P < 0.05$), claudin 28a (*E*; $P < 0.01$), and claudin 30 mRNA (*F*; $P < 0.001$). There was a significant interaction between salinity and time for both plasma osmolality (*D*; $P < 0.05$) and expression of claudin 28a (*E*; $P < 0.01$) and claudin 30 mRNA (*F*; $P < 0.05$). Values are means ± SE (n = 7–10). *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$, significant effects compared with sham controls.

claudins, based on their coding sequence (19–38 kDa; *D. rerio*, Ref. 18; *F. rubripes*, Ref. 23; see Swiss-Prot at http://www.expasy.org/sprot/). Claudin 4-like immunohistochemical staining was found mainly in the epithelial cell layer of the filament, where one would expect to see high concentrations of TJs. The reduced staining in SW gill, limited to a thin layer close to the apical side, suggests that TJs might be thinner in this region. We found claudin 3-like staining deeper in the FW filament relative to that of gill from SW fish. This may not be surprising considering that claudin 3 has been localized to the basolateral membrane of intestinal cells (35). Conversely, in SW gill, claudin 3-like staining is closer to the surface away from the deeper cell layer. The gill epithelium of FW fish is usually considered to be tight due to the deep TJs between adjacent pavement and chloride cells (9, 36). The deeper localization of claudin 3-like proteins and more intense claudin 4-like staining seen in tilapia FW gill, compared with SW gill, may well reflect this. Taken together, the specificity of the antibodies (staining displaceable by claudin 3- and claudin 4-specific peptides), the Western detection of single distinct bands, and their differential localization and regulation in the gill of FW and SW fish suggests that the claudin 3 and claudin 4 heterologous antibodies specifically detect tilapia claudin isoforms and that they may function, in part, in regulating gill permeability associated with changes in environmental salinity.

We examined the dynamics in abundance of putative gill claudins during salinity acclimation. We showed a significant inhibitory effect of SW on gill expression of both claudin 3- and claudin 4-like protein (Fig. 5, *A* and *B*). The decrease in claudin-like proteins became apparent by *day 4* and was most prominent by *day 7* of SW exposure, a downregulation that was accompanied by increases in gill Na^+-K^+-ATPase and Na^+-K^+-2Cl-cotransporter. Therefore, it is likely that a decline in claudin 3- and claudin 4-like proteins is important in reshaping the gill tissue to an ion-secreting and more leaky epithelium. In contrast to that observed with SW acclimation, both claudin 3- and claudin 4-like protein expression were elevated twofold in fish transferred to FW compared with sham SW-transferred controls (Fig. 5, *C* and *D*). These salinity challenge experiments indicate that both claudins show relatively high basal expression levels in the gill of fish, as judged by strong Western blot signals. Furthermore, the remodeling of the gill epithelium from an ion-secreting to an ion-absorptive epithelium was accompanied by enhanced protein expression of the two putative tilapia claudins. It is likely that the elevated claudin 3- and claudin 4-like protein expression in gill of

FW-transferred fish is important in epithelial tightening needed to reduce ion permeability, as is seen in fish that reside in FW environments (29). The differential regulation of claudin proteins seen presently with salinity is consistent with that observed for gene expression of tilapia claudin 28a and 30, which are members of the class VI claudins that are related to the mammalian claudin 3 and 4 (23). We found an upregulation of claudin 28a and claudin 30 mRNAs during FW adaptation (Fig. 7), which is consistent with investigations in the euryhaline European eel, where claudin 27 mRNA levels were higher in FW compared with SW fish (15). By contrast, Boutet et al. (3) sequenced a claudin 3-like gene in the European seabass (homologous to fugu 8d; Ref. 23) and found higher gill mRNA expression of this claudin in fish from the open sea (35 ppt; ~11°C) than in those animals living in a lagoon where salinity fluctuates (3.5–41 ppt) and temperatures are lower (5°C). Whether this gene is regulated in response to temperature changes or salinity specifically is uncertain.

In mammals, increased epithelial expression of claudins is associated with either increased or decreased transepithelial resistance, depending on the isoform. Thus, in a mammalian kidney cell model, it was shown that claudins 8, 11, and 14 decrease paracellular conductance and discriminate against cations, whereas claudins 2 and 15 increase the permeability for Na^+ (20, 44). Claudins also may create charge- and size-selective TJ pores for specific ions, making their potential function in osmoregulation even more complex (1). Since the expression of the two gill claudin isoforms declined during SW transfer and increased in response to FW acclimation, it would appear that the proteins might play a role in TJ modification to provide a tighter or less leaky epithelium.

Perspectives and Significance

Salinity acclimation in euryhaline teleosts involves the dynamic regulation of ion conductance and TJ remodeling. Characterizing the claudin isoforms that are expressed in gills and the corresponding response is critical to understanding how fish adjust to changes in salinity. These studies are the first to detect putative claudin proteins in teleosts and to show that their localization and regulation with salinity in gill epithelium; claudin 3-like and claudin 4-like proteins decline during SW acclimation and are upregulated during FW transfer. In addition, we cloned two tilapia claudin genes and showed that their expression is regulated by salinity in a manner similar to the putative claudin proteins. Overall, the data indicate that claudins may be important in permeability changes associated with salinity acclimation and possibly the formation of deeper tight junctions in FW gill. It will be important in future studies to clone the full suite of branchial claudin isoforms, analyze their expression, and examine their regulation by environmental and hormonal factors.

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GRANTS

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REFERENCES

1. **Alexandre MD, Lu Q, Chen YH.** Overexpression of claudin-7 decreases the paracellular Cl^- conductance and increases the paracellular Na^+ conductance in LLC-PK1 cells. *J Cell Sci* 118: 2683–2693, 2005.
2. **Amasheh S, Meiri N, Gitter AH, Schoneberg T, Mankertz J, Schulzke JD, Fromm M.** Claudin-2 expression induces cation-selective channels in tight junctions of epithelial cells. *J Cell Sci* 115: 4969–4976, 2002.
3. **Boutet I, Long Ky CL, Bonhomme F.** A transcriptomic approach of salinity response in the euryhaline teleost, *Dicentrarchus labrax*. *Gene* 379: 40–50, 2006.
4. **Bustin SA.** Absolute quantification of mRNA using real-time reverse transcription polymerase chain reaction assays. *J Mol Endocrinol* 25:169–193, 2000.
5. **Colegio OR, Van Itallie CM, McCrea HJ, Rahner C, Anderson JM.** Claudins create charge-selective channels in the paracellular pathway between epithelial cells. *Am J Physiol Cell Physiol* 283: C142–C147, 2002.
6. **Cutler CP, Cramb G.** Branchial expression of an aquaporin 3 (AQP-3) homologue is downregulated in the European eel *Anguilla anguilla* following seawater acclimation. *J Exp Biol* 205: 2643–2651, 2002.
7. **D'Souza T, Agarwal R, Morin PJ.** Phosphorylation of claudin-3 at threonine 192 by PKA regulates tight junction barrier function in ovarian cancer cells. *J Biol Chem* 280: 26233–26240, 2005.
8. **Evans DH.** The roles of gill permeability and transport mechanisms in euryhalinity. In: *Fish Physiology*, edited by Hoar WS and Randall DJ. Orlando, FL: Academic, 1984, p. 239–283.
9. **Evans DH, Piermarini PM, Choe KP.** The multifunctional fish gill: dominant site of gas exchange, osmoregulation, acid-base regulation, and excretion of nitrogenous waste. *Physiol Rev* 85: 97–177, 2005.
10. **Furuse M, Sasaki H, Tsukita S.** Manner of interaction of heterogeneous claudin species within and between tight junction strands. *J Cell Biol* 147: 891–903, 1999.
11. **Heiskala M, Peterson PA, Yang Y.** The roles of claudin superfamily proteins in paracellular transport. *Traffic* 2: 92–98, 2001.
12. **Hiroi J, McCormick SD, Kaneko RO, Kaneko T.** Functional classification of mitochondrion-rich cells in euryhaline Mozambique tilapia (*Oreochromis mossambicus*) embryos, by means of triple immunofluorescence staining for Na^+/K^+-ATPase, $Na^+/K^+/2Cl^-$ cotransporter and CFTR anion channel. *J Exp Biol* 208: 2023–2036, 2005.
13. **Hirose S, Kaneko T, Naito N, Takei Y.** Molecular biology of major components of chloride cells. *Comp Biochem Physiol B* 136: 593–620, 2003.
14. **Isaia J, Hirano T.** Effect of environmental salinity change on osmotic permeability of isolated gill of eel, *Anguilla anguilla* L. *J Physiol (Paris)* 70: 737–747, 1976.
15. **Kalujnaia S, McWilliam IS, Zaguinaiko VA, Feilen AL, Nicholson J, Hazon N, Cutler CP, Cramb G.** Transcriptomic approach to the study of osmoregulation in European eel *Anguilla anguilla*. *Physiol Genomics* 31: 385–401, 2007.
16. **Kamiya M, Utida S.** Sodium-potassium-activated adenosinetriphosphatase activity in gills of fresh-water, marine and euryhaline teleosts. *Comp Biochem Physiol* 31: 671–674, 1969.
17. **Karnaky KJ Jr.** Teleost osmoregulation: changes in the tight junction in response to the salinity of the environment. In: *TightJunctions*, edited by Cereijido M. Boca Raton, FL: CRC, 1991, p.175–185.
18. **Kollmar R, Nakamura SK, Kappler JA, Hudspeth AJ.** Expression and phylogeny of claudins in vertebrate primordia. *Proc Natl Acad Sci USA* 98: 10196–10201, 2001.
19. **Laurent P.** Gill internal morphology. In: *Fish Physiology*, edited by Hoar WS and Randall DJ. Orlando, FL: Academic, 1984, p.73–183.
20. **Li WY, Huey CL, Yu ASL.** Expression of claudin-7 and -8 along the mouse nephron. *Am J Physiol Renal Physiol* 286: F1063–F1071, 2004.
21. **Lignot JH, Cutler CP, Hazon N, Cramb G.** Immunolocalisation of aquaporin 3 in the gill and the gastrointestinal tract of the European eel *Anguilla anguilla* (L.). *J Exp Biol* 205: 2653–2663, 2002.
22. **Lin CH, Huang CL, Yang CH, Lee TH, Hwang PP.** Time-course changes in the expression of Na, K-ATPase and the morphometry of mitochondrion-rich cells in gills of euryhaline tilapia (*Oreochromis mossambicus*) during freshwater acclimation. *J Exp Zool* 301A: 85–96, 2004.
23. **Loh YH, Christoffels A, Brenner S, Hunziker W, Venkatesh B.** Extensive expansion of the claudin gene family in the teleost fish, *Fugu rubripes*. *Genome Res* 14: 1248–1257, 2004.

24. **Lorin-Nebel C, Boulo V, Bodinier C, Charmantier G.** The $Na^+/K^+/2Cl^-$ cotransporter in the sea bass *Dicentrarchus labrax* during ontogeny: involvement in osmoregulation. *J Exp Biol* 209: 4908–4922, 2006.
25. **Lu G, Moriyama EN.** Vector NTI, a balanced all-in-one sequence analysis suite. *Brief Bioinform* 5: 378–388, 2004.
26. **Lytle C, Xu J, Biemesderfer D, Forbush IIIB.** Distribution and diversity of Na-K-Cl cotransport proteins: a study with monoclonal antibodies. *Am J Physiol Cell Physiol* 269: C1496–C1505, 1995.
27. **Marshall WS.** Transepithelial potential and short-circuit current across the isolated skin of *Gillichthys mirabilis* (Teleostei: Gobiidae) acclimated to 5% and 100% seawater. *J Comp Physiol [B]* 114: 157–165, 1977.
28. **Marshall WS, Bryson SE, Darling P, Whitten C, Patrick M, Wilkie M, Wood CM, BucklandNicks J.** NaCl transport and ultrastructure of opercular epithelium from a freshwater-adapted euryhaline teleost, *Fundulus heteroclitus*. *J Exp Zool* 277: 23–37, 1997.
29. **Marshall WS, Emberley TR, Singer TD, Bryson SE, McCormick SD.** Time course of salinity adaptation in a strongly euryhaline estuarine teleost, *Fundulus heteroclitus*: a multivariable approach. *J Exp Biol* 202: 1535–1544, 1999.
30. **McCormick SD.** Endocrine control of osmoregulation in teleost fish. *Am Zool* 41: 781–794, 2001.
31. **Morgan JD, Sakamoto T, Grau EG, Iwama GK.** Physiological and respiratory responses of the Mozambique tilapia (*Oreochromis mossambicus*) to salinity acclimation. *Comp Biochem Physiol A* 117: 391–398, 1997.
32. **Pelis RM, Zydlewski J, McCormick SD.** Gill $Na^+,K^+,2Cl^-$ cotransporter abundance and localization Atlantic salmon: effects of seawater and smolting. *Am J Physiol Regul Integr Comp Physiol* 280: R1844–R1852, 2001.
33. **Picha ME, Silverstein JT, Borski RJ.** Discordant regulation of hepatic IGF-I mRNA and circulating IGF-I during compensatory growth in a teleost, the hybrid striped bass (*Morone chrysops* × *M. saxatilis*). *Gen Comp Endocrinol* 147:196–205, 2006.
34. **Powell DW.** Barrier function of epithelia. *Am J Physiol Gastrointest Liver Physiol* 241: G275–G288, 1981.
35. **Rahner C, Mitic LL, Anderson JM.** Heterogeneity in expression and subcellular localization of claudins 2, 3, 4, and 5 in the rat liver, pancreas, and gut. *Gastroenterology* 120: 411–422, 2001.
36. **Sardet C, Pisam M, Maetz J.** The surface epithelium of teleostean fish gills. Cellular and junctional adaptations of the chloride cell in relation to salt adaptation. *J Cell Biol* 80: 96–117, 1979.
37. **Seidelin M, Madsen SS, Blenstrup H, Tipsmark CK.** Time-course changes in the expression of Na^+,K^+-ATPase in gills and pyloric caeca of brown trout (*Salmo trutta*) during acclimation to seawater. *Physiol Biochem Zool* 73: 446–453, 2000.
38. **Sonoda N, Furuse M, Sasaki H, Yonemura S, Katahira J, Horiguchi Y, Tsukita S.** Clostridium perfringens enterotoxin fragment removes specific claudins from tight junction strands: evidence for direct involvement of claudins in tight junction barrier. *J Cell Biol* 147: 195–204, 1999.
39. **Takeyasu K, Tamkun MM, Renaud KJ, Fambrough DM.** Ouabain-sensitive $(Na^+ + K^+)$-ATPase activity expressed in mouse L cells by transfection with DNA encoding the α subunit of the avian sodium pump. *J Biol Chem* 263: 4347–4354, 1988.
40. **Tatusova TA, Madden TL.** BLAST 2 Sequences—a new tool for comparing protein and nucleotide sequences. *FEMS Microbiol Lett* 174: 247–250, 1999.
41. **Tipsmark CK, Madsen SS, Seidelin M, Christensen AS, Cutler CP, Cramb G.** Dynamics of $Na^+,K^+,2Cl^-$ cotransporter and Na^+,K^+-ATPase expression in the branchial epithelium of brown trout (*Salmo trutta*) and Atlantic salmon (*Salmo salar*). *J Exp Zool* 293: 106–118, 2002.
42. **Tipsmark CK, Luckenbach JA, Madsen SS, Borski RJ.** IGF-I and branchial IGF receptor expression and localization during salinity acclimation in striped bass. *Am J Physiol Regul Integr Comp Physiol* 292: R535–R543, 2007.
43. **Turksen K, Troy TC.** Barriers built on claudins. *J Cell Sci* 117: 2435–2447, 2004.
44. **Van Itallie CM, Anderson JM.** Claudins and epithelial paracellular transport. *Annu Rev Physiol* 68: 403–429, 2006.
45. **Wilson JM, Laurent P, Tufts BL, Benos DJ, Donowitz M, Vogl AW, Randall DJ.** NaCl uptake by the branchial epithelium in freshwater teleost fish: an immunological approach to ion-transport protein localization. *J Exp Biol* 203: 2279–2296, 2000.
46. **Wilson JM, Antunes JC, Bouca PD, Coimbra J.** Osmoregulatory plasticity of the glass eel of *Anguilla anguilla*: freshwater entry and changes in branchial ion-transport protein expression. *Can J Fish Aquat Sci* 61: 432–442, 2004.
47. **Wu YC, Lin LY, Lee TH.** $Na^+,K^+,2Cl^-$ cotransporter: a novel marker for identifying freshwater- and seawater-type mitochondria-rich cells in gills of the euryhaline tilapia, *Oreochromis mossambicus*. *Zool Stud* 42: 186–192, 2003.
48. **Yoshikawa JSM, McCormick SD, Young G, Bern HA.** Effects of salinity on chloride cells and Na^+-K^+-ATPase activity in the teleost *Gillichthys mirabilis*. *Comp Biochem Physiol A* 105: 311–317, 1993.