学位論文

Functional differentiation of chloride metabolism in euryhaline tilapia, *Oreochromis mossambicus*
広塩性魚ティラピアのクロライド代謝系の機能分化

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Functional differentiation of chloride metabolism in euryhaline tilapia, *Oreochromis mossambicus*

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by

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General Introduction

A majority of teleost fishes confine their life to the sea while others spend their entire life in inland fresh water (FW). There are some species, however, which spend a part of their life in both FW and seawater (SW). Unlike those living entirely in SW or FW, fishes which spend their life in both environments face the problem of regulating water and ion contents of their body fluid to opposite directions when exposed to different salinities. The regulation of water and ion composition in fish takes place in the gills, kidney and intestine. Mechanisms of teleost osmoregulation have been described extensively in several reviews (Evans, 1979, 1993; Rankin and Davenport, 1981). Briefly, SW fishes lose water and gain ions through the body surfaces, mainly through the gills. In order to compensate the osmotic loss of water, they drink surrounding SW and absorb both water and ions from the intestine. The excess Na^+ and Cl^-, which enter the body through the gills and intestine, are excreted actively by the gills. By contrast, FW fishes gain water and lose ions through the gills. Thus, they scarcely drink water and produce a large amount of hypotonic urine. The renal losses of ions and the passive loss across the body surface are compensated by an active uptake of ions by the gills.

The fish gills, like any other gas-exchange organs, are modified to (1) maximize the surface area available for exchange of O_2 and CO_2, (2) minimize the epithelial height to promote the diffusion of gases between the external medium and the

blood, and (3) maximize the blood perfusion of the tissue. The gills evolved from the surface epithelia of the branchial basket of proto-vertebrates, probably about 550 million years ago in the *Pteraspid* agnathans (Gilbert 1993). Evolutionary modification of the surface epithelium to facilitate gas exchange is at physiological costs in an aquatic environment since it exacerbates any diffusional movement of solutes or water into or out of the organism where body fluid is not iso-osmotic to the medium. Ancestors of the vertebrates have their plasma iso-osmotic and iso-ionic to their marine environments; however, for reasons that are still debated (Griffith 1987, Evans 1993), all vertebrates except the hagfish have their plasma different from SW. Thus the facilitated gas exchange poses in aquatic environments osmoregulatory problems to fishes. Although osmoregulation in fishes is achieved by a suite of structures including the gut and kidney, the gill is the major site of water and ion movements to balance their diffusional gains and losses.

A FW fish, when it enters SW, faces the problem of salt loading and water loss. By contrast, when a SW fish enters FW, it has to solve the problem of ion loss and water loading. The fact that Na^+ and Cl^- concentration in fish plasma differ from that of either FW or SW has been known for more than 100 years (Holmes and Donaldson 1969). The large Na^+ and Cl^- gradients across the gills of FW fishes could not be maintained by the electrical potentials that have been measured across the FW body surfaces (Evans 1980, Potts 1984, Potts and Hedge 1991). Therefore, it is clear that

net diffusional loss of Na^+ and Cl^- should occur in these fishes. This branchial loss and the renal loss of Na^+ and Cl^- must be counterbalanced by some sort of Na^+ and Cl^- uptake system. Even with these osmoregulatory problems, euryhaline fishes should be able to maintain their internal water and ion concentrations at a constant level in both FW and SW.

About 60 years ago, Keys and Wilmer (1932) suggested that a certain type of gill epithelial cells might be responsible for Cl^- excretion in SW-adapted eel. Later, Copeland (1948) described the cells, presumably for the first time, as "chloride cells" in FW- and SW-adapted killifish, *Fundulus heteroclitus*. These highly specialized cells are characterized by numerous mitochondria, a large columnar appearance and apical exposure to the environments. The chloride cell also contains an extensive membranous tubular system in the cytoplasm which is continuous with the basolateral membrane.

The sites of active extrusion of Na^+ and Cl^- in adult teleosts in SW are the chloride cells (see Zadunaisky 1984). Several models have been proposed for the mechanisms of active ion transport across the gill epithelium, including specific routes through chloride cells in fish adapted to either FW and SW (Towle 1990; Lin and Randall 1995; Marshall 1995). Many of the models predict differences in the composition or distribution of ion channels and transporters in the chloride cells between FW- and SW-adapted fish.

A model of ion-transporting system in the chloride cells of SW fish is shown in Fig. 1, which is a modification of the model proposed initially by Silva *et al.* (1977). The ultimate driving force for Cl^- secretion is an electrochemical gradient of Na^+ established by Na^+, K^+-ATPase located on the basolateral membrane. Cl^- enters the cell across the basolateral membrane through Na^+-dependent Na^+, K^+, $2Cl^-$ cotransporter (Marshall 1981, Degnan 1984). Cl^- accumulates intracellularly while cytoplasmic electric potential is lower than the environment so that Cl^- exit occurs down its electrochemical gradient through anion channels in the apical membranes. Na^+ secretion occurs down its electrochemical gradient via cation-selective paracellular pathway (Degnan and Zadunaisky 1980, Marshall 1981) that is located apically between chloride cells the smaller adjacent (or accessory) cells (Sardet et al. 1979, Hootman and Philpott 1980).

The models of ion uptake mechanisms in FW include electrically silent anion and cation exchangers that allow uptake of Na^+ and Cl^- in roughly equivalents depending on the relative speed of operation of the two exchangers (Fig. 1). Classically, the Na^+/H^+ exchanger accepts NH_4^+ instead of H^+ when ammonia is present, and takes up Na^+ in exchange of NH_4^+ (Maetz and Garcia Romeu 1964). The transport is dependent on the cytosolic carbonic anhydrase and on the basolaterally located Na^+, K^+-ATPase. The "active" step for Cl^- transport occurs almost certainly at the apical membrane despite the large electrochemical gradient operating against Cl^-

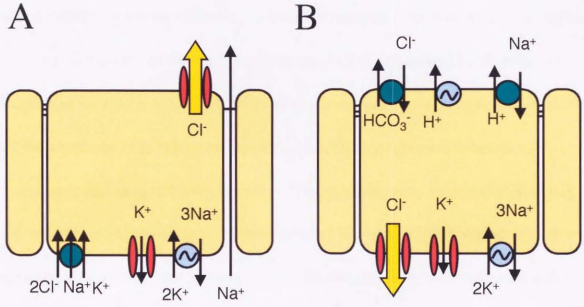


Fig. 1 (A) Diagrammatic summary of a model of chloride cell function in SW-adapted fish. "Leaky" intercellular junctions are single strands; "tight" intercellular junctions are multi strands.

(B) A model of chloride cell function in FW-adapted fish.

uptake; the Cl^- uptake may be driven by an HCO_3^- gradient produced by the carbonic anhydrase and the subsequent obligatory electroneutral exchange with HCO_3^- by Cl^- /HCO_3^- exchangers. A second mechanism, apical membrane H^+-ATPase, would help create a negative electrochemical gradient intracellularly to drive Na^+ uptake across the apical membrane via Na^+ channels. These ion transport models of the chloride cell in FW and SW expect that the transport direction of Cl^- is determined by the cellular location of the chloride channels. Therefore, ion-regulating mechanisms are controlled by the regulation of a number of transporters located both apical and basolateral membranes including chloride channels. Since chloride cells are established to play pivotal roles in ion uptake and excretion in both FW and SW fishes, it is obvious that the gill is a most important osmoregulatory site in maintaining ionic balance in fish.

The aim of the experiments presented in this thesis is to examine the process of functional differentiation of ion regulation of the gill in fish adapted either to FW or to SW. Although a large majority of teleosts survive only in FW or SW, some euryhaline fish such as the eel, salmon and tilapia do in both media. Among members of the family Cichlidae that are generally referred to as tilapias, *Oreochromis mossambicus* is one of the most salinity-tolerant species, reproducing both in FW and SW. Therefore, it provides a good model to examine the functional differentiation of ion regulation in the gill.

In Introductory Chapter, changes in Cl^- permeability during development were

examined in early life stages of tilapia to confirm the developmental changes of ion-regulating functions of the gills. Changes in ion permeability were examined after transfer of the embryos from FW to SW. The diffusional Cl^- permeability remained low during embryonic stages but increased markedly after hatching in both FW and SW. At hatching, owing to the formation of the gill structures including filament and lamellae, there is a large increase in surface area of the body. Gray (1954) has shown that the gill of adult teleosts occupies most surface area of the whole fish. He found that in 17 species of teleosts the gill surface area alone is 1.3-18.3 times wider than the remaining surface area of the body. The increase in the turnover rate of Cl^- at hatching might be attributed to the increase in the surface area of the gill. Therefore, it is apparent that the gill is the most important organ that is concerned in Cl^- exchange between the body fluid and external environment. The turnover of Cl^- in SW was 50-100 times greater than that in FW. It is highly probable that the physico-chemical properties of the gill epithelia in FW larvae are different from those in SW ones.

In Chapter 1, with an assumption that the large increase in Cl^- turnover in SW fish obtained in the experiments of Introductory Chapter is due to an increase in chloride channels, we attempted to identify chloride channels in tilapia gill using molecular biological technique. Identification of chloride channels is essential for fully understanding the Cl^- transport regulation at the cell level in the subsequent physiological studies of Cl^- transport regulation. Two clones homologous to

mammalian CLC chloride channels were isolated from a gill cDNA library. These cDNAs encoded two protein products which were named OmCLC-3 and OmCLC-5. The amino acid sequence of OmCLC-3 was 90.5% identical to rat CLC-3, and OmCLC-5 was 79.2% identical to rat CLC-5. Ribonuclease protection analysis revealed that the mRNA of OmCLC-5 was expressed mainly in the gill, kidney and intestine, whereas the mRNA of OmCLC-3 was expressed ubiquitously in almost all tissues examined. These results suggest that OmCLC-5 may be important for osmoregulation in the tilapia. As OmCLC-5 might be involved in osmoregulation of tilapia, changes in the expression of its mRNA were examined in the gill in response to changes in environmental salinity. The results indicate that the expression level of OmCLC-5 was increased in hypertonic media and the increase became significant in fish adapted to double-strength (2x) SW compared with FW controls.

In Chapter 2, since it is not possible to identify the clones as chloride channels only from the structural similarity, the function of OmCLC-3 and OmCLC-5 was examined by expression of the clones. In the first experiment, we expressed OmCLC-3 and OmCLC-5 in *Xenopus* oocyte and the Cl^- conductance of each channel was examined electrophysiologically by a whole-cell-clamp technique. Both OmCLC-3 and OmCLC-5 expressed in *Xenopus* oocytes generated outwardly rectifying Cl^- current. Although the generated currents were significantly inhibited by chloride channel blocker DIDS, these currents were too small to manifest the clear inhibition

compared with mammalian CLC-3 and CLC-5 (about 1/50-1/100). In the second experiment, the function of OmCLC-3 and OmCLC-5 as chloride channel was examined using a yeast strain in which only one CLC channel (GEF1) present in the yeast has been disrupted by homologous recombination. The OmCLC-3 and OmCLC-5 expressed in the yeast did functionally complement the gef1 phenotype of the genetically CLC-deficient strain. This result clearly shows that the tilapia CLC channels have the functions of CLC chloride channel family.

In the last Chapter, in order to examine the osmoregulatory function of OmCLC-5 that is expressed specifically in osmoregulatory organs, cellular localization of OmCLC-5 was examined in the gills of FW- and 2xSW-adapted tilapia using immunocytochemical techniques at both light- and electron-microscopic levels. As shown in Fig. 1, the localization of the channels is important for the full expression of its function. It was found that OmCLC-5 was localized in specific cells of the gills. In comparison with the localization of Na^+, K^+-ATPase, OmCLC-5 was found to localize specifically in the chloride cells. Electron microscopic immunocytochemistry revealed that the staining of OmCLC-5 was observed in the basolateral side of plasma membranes of chloride cells including the tubular system. The specific localization of OmCLC-5 in the chloride cells, together with its function as a chloride channel, strongly indicates that OmCLC-5 function as an important chloride transporter in the chloride cells. Furthermore, since OmCLC-5 expression increases in hypertonic environments,

this chloride channel is likely to be responsible for enhanced Cl^- turnover in SW as shown in this thesis.

Introductory Chapter

Developmental changes in chloride ion permeability during early life stages of tilapia, *Oreochromis mossambicus*

Abstract

Developing embryos and larvae of euryhaline tilapia (*Oreochromis mossambicus*) can withstand direct transfer from fresh water (FW) to seawater (SW) or *vice versa* before development of osmoregulatory organs such as the gills. In the present study, developmental changes in ion permeability were compared between late embryonic and early larval stages of tilapia reared in FW and SW. While turnover rates of Cl^- in FW larvae 1 day before hatching were 0.002 h^{-1}, the rates of SW tilapia were 0.19 h^{-1}. Therefore, the turnover rates of Cl^- were about 100 times greater in SW fish than in FW fish. Furthermore, the rates increased markedly after hatching in both FW and SW. The values of FW- and SW-adapted tilapia larvae were comparable to those reported in stenohaline species.

Introduction

Teleost fishes maintain ionic composition and osmolarity of the body fluid at levels different from those of external environments by adjusting the fluxes of water and ions at the osmoregulatory surfaces (see Evans 1979, 1984). In adult fish, the gills, kidney and intestine are important osmoregulatory organs, creating ionic gradients between the body fluid and external environments. Although these osmoregulatory organs are not fully developed in early life stages of fish, the eggs and embryos of both freshwater (FW) and marine teleosts are also able to maintain the osmotic gradient (see Holliday 1969; Alderdice 1988).

A number of teleost species are euryhaline, but their spawning ground is generally restricted either to FW or to seawater (SW), as is typically the case for migratory species such as eels and salmonids. Among members of the family Cichlidae that are generally referred to as tilapias, *Oreochromis mossambicus* is one of the most salinity-tolerant species, reproducing both in FW and in SW (Stickney 1986; Suresh and Lin 1992). Furthermore, direct transfer of fertilized eggs, developing embryos or newly hatched larvae from FW to SW, or *vice versa*, is possible without mortality (Kwaye et al. 1992; Ayson et al. 1994). Thus, FW- and SW-adapted embryos and larvae from the same brood are readily available for experiments, providing a suitable model for studies of osmoregulatory mechanisms during early life stages.

In our previous study, we demonstrated a rich population of mitochondria-rich chloride cells in the yolk-sac membrane of tilapia embryos and larvae (Ayson et al. 1994). These chloride cells are highly activated in SW, suggesting their important roles in ion excretion in SW in place of the gill chloride cells. The regulation of ion balance during early embryonic stages seems to be achieved in part by extremely low permeability of the body surface.

To further explore the functional differentiation of osmoregulatory mechanisms during embryonic and larval stages of tilapia, developmental changes in Cl^- permeability were examined in FW- and SW-adapted embryos and larvae originated from the same brood. The results clearly showed that the ion turnover rate of Cl^- is affected dramatically by the environmental salinity and during the developmental stages.

Materials and methods

Fish

Adult tilapia, *Oreochromis mossambicus*, were collected from a FW pond in northern Okinawa Island, and maintained in recirculating FW tanks at 25 °C. They were kept for breeding in 200-l tanks and fed on compound tilapia pellets, "Tilapia 41M" (Shikoku Kumiai Shiryo, Tokushima, Japan) once a day. Embryos were obtained from the mouth of a brooding female 3 days after fertilization. They were separated into two groups; one half of the embryos were maintained in FW and the other half were transferred directly to SW (salinity, 33.5 ‰). Tilapia embryos usually hatched 5 days after fertilization at 25 °C, and the yolk absorption was completed by 10 days after hatching. The day of hatching was designated as day 0.

Chloride permeability

The rate of Cl^- uptake was measured by whole-body influx of $^{36}Cl^-$ as described by Guggino (1980). Twenty-five embryos on day -1 and larvae on days 2, 6 and 10 were placed in a beaker containing 15 ml FW or SW. The FW-adapted embryos and larvae were sampled at 0.5, 1, 2, 3 and 6 h after adding $Na^{36}Cl$ (4.37 MBq ml^{-1}, Amersham Pharmacia Biotech, Uppsala, Sweden) to give a final specific activity of 30 KBq ml^{-1}. The changes in Cl^- levels of the bathing media osmolarity were negligible (0.01 mOsm/kg). Since turnover rates of SW-adapted embryos and larvae were assumed to be higher than that of FW fish, shorter sampling intervals were adopted; fish

were sampled at 15, 30, 45, 60, 75, 90, 105, 120, 150 and 180 min after addition of $Na^{36}Cl$. Five fish in FW or one fish in SW were removed from the beaker using a wide-mouthed pipette, transfer into a stainless steel basket and washed 5 times (1 min each). The embryos or larvae were anesthetized and wet weights of the embryos and larvae were determined after removing excess water with Kim wipe tissue (Kimberly-Clark Worldwide, Inc., Neenah, WI, U.S.A.). Then the embryos and larvae were placed in a miniature scintillation vial containing 1 ml solubilizing agent (Soluene-350, Packard, Meriden, CT, U.S.A.). When they were completely solubilized (24 h at 25 °C), 3 ml scintillation fluid (Hionic Fluor, Packard) was added. After solubilization, radioactivity was measured using a liquid scintillation counter (LS6000SC, Beckman, Fullerton, CA, U.S.A.). The time course of uptake of $^{36}Cl^-$ resulted in a nonlinear curve (Fig. 2). The rate of Cl^- uptake was analyzed using a first-order rate equation as described by Brown and Tytler and Ireland (1993):

$$Q=Qeq\ (1-e^{-kt}),$$

where Q (dpm) is the radioactivity in the fish at time t, Qeq (dpm) is the equilibration level of radioactivity in the fish and k is the rate constant of uptake.

Statistics

Significant differences were determined by Cochran-Cox test or Student t-test after analysis of variance. Significance was determined at $P<0.05$. Values are mean ± SEM.

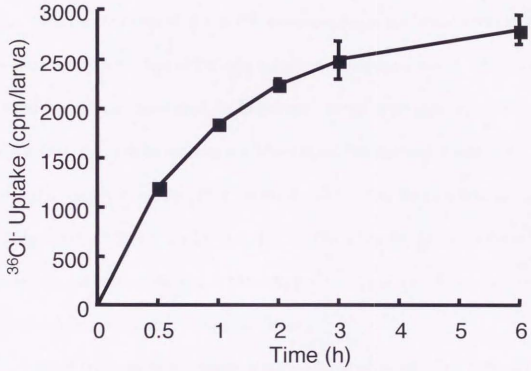


Fig. 2 Time course of Cl^- uptake measured by accumulation of $^{36}Cl^-$ in tilapia larvae 6 days after hatching. Each point represents the mean $\pm$ SEM (n=3). Error bars are omitted in case where the values are within the symbols.

Results

In the present study, tilapia embryos 2 days before hatching (3 days after fertilization) were transferred directly from FW to SW, and no mortality was seen during the experiment.

The turnover rates of $^{36}Cl^-$ in FW-adapted embryos and larvae were extremely low as compared with those of SW-adapted fish at all stages examined. Similar to the turnover rate of water, however, Cl^- turnover rates increased after hatching in both FW and SW (Fig. 3). The turnover rates in SW-adapted fish increased 4 fold from $0.2\pm0.03\ h^{-1}$ on day -1 to $0.8\pm0.02\ h^{-1}$ on day 2. In FW fish, the rates also increased 15 fold from $0.001\pm0.0001\ h^{-1}$ on day -1 to $0.015\pm0.004\ h^{-1}$ on day 2. The rates on days did not increase further thereafter in both fish groups. Thus, the Cl^- turnover rates were maintained at rather constant levels after hatching.

From these results, it is apparent that (1) the turnover rate of Cl^- is much higher in the embryos and larvae of SW tilapia than in FW fish, and (2) the rate dramatically increases after hatching.

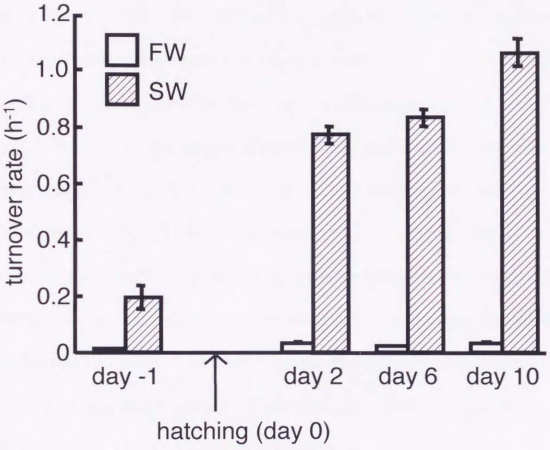


Fig. 3 Changes in turnover rate of Cl^- for tilapia embryos and larvae adapted to FW and SW. Each point represents the mean $\pm$ SEM (n=3). FW level of Cl^- turnover rate on day -1 and 6 were too low to shown in figure (0001 $\pm$ 0.0001 and 0.0087 $\pm$ 0.0003 h^{-1}, respectively).

Discussion

In the present study, tilapia embryos 2 days before hatching (3 days after fertilization) were transferred directly from FW to SW, and no mortality was seen during the experiment, indicating their strong euryhalinity even before the development of osmoregulatory organs such as the gills, kidney and intestine.

The turnover rates for Cl^- increased markedly at hatching in both FW and SW fish. Similarly, the rate of Cl^- turnover increased at hatching in *Fundulus heteroclitus* and *F. berumudae* in SW (Guggino 1980). The increase was somewhat grater in the killifish (from 0.026 h^{-1} to 2.0 h^{-1}) than in the tilapia (0.18 h^{-1} to 0.78 h^{-1}). The increased Cl^- turnover may be in part due to an increase in the surface area owing to the formation of adult gill structures. However, the fact that Cl^- turnover increased about 4 times at hatching in SW and 15 times in FW may be difficult to be explained only by an increase in newly-formed gill surfaces. Furthermore, Cl^- turnover rates of tilapia embryos and larvae in SW were markedly higher than those in FW. Similar differences in Na^+ turnover occur in adult tilapia (0.03 h^{-1} in FW and 0.66 h^{-1} in SW) and in many other euryhaline teleosts (Potts et al., 1967; Maetz 1974). It is highly probable that the physico-chemical properties of the body surface epithelia in FW larvae are different from those in SW larvae. In adult fish, there is a strong correlation between active uptake of Cl^- and water in the gut (Skadhauge 1974; Kirsch and Meister 1982). If the same mechanisms operate in the larval gut, the high Cl^- turnover in SW

larvae could be due in part to a functional difference from the gut of FW larvae.

The sites of active extrusion of Na^+ and Cl^- in adult teleosts in SW are the chloride cells located mainly in the gills (Zadunaisky 1984). Ayson et al. (1994) reported that after transfer of newly-hatched tilapia larvae from FW to SW, the size of chloride cells in the yolk-sac membrane increased markedly, although the density of the cells was not significantly altered. The difference in Cl^- permeability between FW- and SW-adapted tilapia embryos and larvae in this study may be related in part to the changes in the chloride cell morphology. In general, FW chloride cells are joined to the surrounding epithelial cells by tight junctions at their apical margins (Zadunaisky 1984). The more elaborate tight junctions may be related to the observed lower ionic permeability of the tilapia embryos and larvae in FW. In SW, there is an elaboration of chloride cells and accessory cells, whose interdigitation of basolateral membranes provides a paracellular pathway that is "leaky" (Marshall 1995).

Several models have been proposed for the mechanisms of active ion transport across the gill epithelium, including the specific routes through chloride cells of FW and SW fish (Towle 1990; Lin and Randall 1995; Marshall 1995). Many of the models predict differences in the composition or distribution of ion channels and transporters in the gill epithelia and chloride cells between FW- and SW-adapted fish. To my knowledge, however, it remains unknown whether or not the "leaky" junction between chloride cells and accessory cells are the main route for ion exchange in SW teleosts.

The embryos and larvae of euryhaline tilapia should provide a good model to study functional development of osmoregulatory surfaces as well as, more specifically, to examine how the regulatory mechanism of water and ion channels and transporters are modulated in the osmoregulatory surfaces in response to changes in the environmental salinity.

Chapter 1

Molecular cloning of chloride channels from the gills of tilapia

Abstract

We have cloned two members of the CLC chloride channel family, named OmCLC-3 and OmCLC-5, from the gill cDNA libraries of tilapia *Oreochromis mossambicus*. These cDNAs encode two protein products, the amino acid sequences of which had high homologies to rat CLC-3 and CLC-5. OmCLC-3 was 90.5% identical to rat CLC-3 and OmCLC-5 was 79.2% identical to rat CLC-5. Ribonuclease protection analysis revealed that mRNA of OmCLC-5 was expressed mainly in the gill, kidney and intestine in SW-adapted tilapia, while the mRNA of OmCLC-3 was expressed ubiquitously in all tissues examined. These results suggest that tilapia OmCLC-5 may be involved in osmoregulation of this fish species.

In order to evaluate the role of OmCLC-5 in the osmoregulation of tilapia, changes in expression level of its mRNA was examined in the gill after changes in environmental salinities using ribonuclease protection assay. The results indicate that the expression of OmCLC-5 was increased in hypertonic environments and the difference was significant between 2xSW-adpted fish and FW-adapted fish.

Introduction

Chloride channels are passive anion transport proteins involved in basic functions common to all cells such as the regulation of cell volume and intravesicular pH. In mammals, chloride channels are important for the transepithelial chloride transport in osmoregulatory organs and the regulation of excitability of muscle and nerve. The CLC chloride channel family, originally discovered in the electric ray (*Torpedo*) (Jentsch et al., 1990), includes of 9 members in mammals. A member of the family is also found in bacteria (Fujita et al., 1994), yeast (Greene et al., 1993), plants (Lurin et al., 1996; Hechenberger et al., 1996) and amphibians (Limdenthal et al., 1997). The CLC-1 is established as a skeletal muscle chloride channel that controls the contraction of the muscle fiber (Steinmeyer et al., 1991). The ubiquitously expressed CLC-2 can be activated by cell swelling in oocytes and possibly plays a role in the regulation of cell volume (Thiemann et al., 1992). The CLC-K1 and CLC-K2 channels are exclusively expressed in the kidney and involved in transepithelial chloride transport (Uchida et al., 1993; Kieferle et al., 1994; Adachi et al., 1994). The CLC-3 (Kawasaki et al., 1994), CLC-4 (van Slegtenhorst et al., 1994), and CLC-5 (Fisher et al., 1995; Sakamoto et al., 1996) constitute a subfamily of this gene family. The CLC-3 is recently shown to be a volume-regulating chloride channel (Duan et al., 1997), and the mutations lacking functional human CLC-5 were shown to result in X-linked recessive nephrolithiasis (Lloyd et al., 1997). The only CLC homologue in the yeast, GEF1, is

most closely related to this sub-branch.

As a first step to identify chloride channels responsible for increased Cl^- transport in SW as shown in the Introductory Chapter, we sought to isolate CLC chloride channels in the euryhaline tilapia, *Oreochromis mossambicus* reared in both FW and SW. We chose the gill to construct a cDNA library because this organ plays an essential role in SW adaptation in both larvae and adult tilapia as suggested in the previous chapter. We isolated two cDNA encoding CLC chloride channels and named OmCLC-3 and OmCLC-5 based on the sequence similarities to the previously cloned mammalian counterparts.

Materials and Methods

Fish

Adult tilapia, *Oreochromis mossambicus*, were collected from a freshwater (FW) pond in northern Okinawa Island and maintained in circulating FW tanks at 25^{o}C. To prepare seawater (SW)-adapted fish, some of the fish were transferred to 50 % SW for 2 days and then to 100% SW for at least 30 days. To prepare 2xSW-adapted fish, some of the SW-adapted fish were transferred to 150% SW for 7 days and then to 200% SW for at least 30 days.

Tissue preparation

Tilapia adapted to different salinities (weighing 15-20g) were anesthetized with 0.05% 2-phenoxyethanol. They were sacrificed by decapitation and gills were dissected out. Total RNA, used for reverse transcription (RT)-PCR and for construction of the cDNA library, was extracted from the gill of both FW and SW-adapted tilapia using TRIZOL Reagent (GIBCO BRL Life Technologies Inc., Rockville, MD, U.S.A.).

Reverse transcription RT-PCR

For RT-PCR, we made degenerate primers that corresponded to the conserved sequences in the CLC families: sense strand, CCGGATCCTCNGGN(A/T/C)TNCCNGA(A/G)NTNAA; antisense strand, CCGAATTCTGNA(C/T)NA(G/A/T)NGGNCCN(A/T)(G/A/C)(C/T)TTNCC. One

microgram of total RNA from the gills of FW- and SW-adapted tilapia was reverse-transcribed using avian myelopblastosis virus reverse transcriptase (Roche Diagnostics, Indianapolis, IN, U.S.A.) at 42 ^{O}C for 60 min and then heated at 94 ^{O}C for 5 min. The synthesized cDNA was used for subsequent PCR using synthesized primers in the following profile: 94^{O}C for 30 sec, 55 ^{O}C for 30 sec, 72 ^{O}C for 30 sec, 30 cycles. The PCR products were cut with *Eco* RI and *Bam* HI on both ends, ligated into *Eco* RI and *Bam* HI-cut pSPORT1 (GIBCO BRL Life Technologies Inc., Rockville, MD, U.S.A.), and then sequenced.

Library construction and screening

Oligo (dT)-primed directional tilapia gill (FW- and SW-adapted) cDNA libraries in λZipLox expression vector (GIBCO BRL Life technology Inc., Rockville, MD, U.S.A.) were constructed by the SUPERSCRIPT λSYSTEM for the cDNA Synthesis kit (GIBCO BRL Life Technology Inc., Rockville, MD, U.S.A.). Two PCR products that have 150 bp inserts homologous to mammalian CLC chloride channels were screened from these libraries. The full-length clones (4.2 kbp and 3.8 kbp, respectively) obtained from the cDNA libraries were subcloned into pZL1 (GIBCO BRL Life technology Inc., Rockville, MD, U.S.A.), and nested deletion clones prepared with Erase-A-Base System (Promega, Madison, WI, U.S.A.) were sequenced.

Ribonuclease (RNase) protection assay

For the detection of expression of each clone, PCR clones used for the library

screening were linearized and used to prepare radiolabeled antisense RNA probes. These probes (1×10^5 cpm/sample) were mixed with 20 μg of total RNA from gill, kidney, intestine, liver, spleen, muscle, and brain of FW- and SW-adapted tilapia. After hybridization and subsequent RNase digestion, protected fragments were analyzed by electrophoresis in 6% denaturing polyacrylamide gel (RPA II Kit, Ambion, Austin, TX, U.S.A.).

Expression of OmCLC-3 and OmCLC-5 in the osmoregulatory organs

As cloned CLCs were expressed in osmoregulatory organs and thus might be involved in osmoregulation of tilapia, changes in the expression of their mRNA were examined in the gill in response to change in environmental salinity by RNase protection assay. In the case of OmCLC-5, the expression was also examined in double strength SW (2xSW) fish. We used tilapia β-actin as a probe (1×10^5 cpm/sample, 210 bp) to normalize for the amount of RNA assayed. The hybridization signals for both OmCLCs and tilapia β-actin were quantified by densitometric scanning of autoradiographs (Fuji BAS-2000; Fuji Film, Tokyo, Japan). Densities of OmCLCs were normalized to the density of corresponding β-actin.

Statistics

Significant differences were determined by Cochran-Cox test or Student t-test after examination by analysis of variance. Significance was determined at $P<0.05$. Values are mean ± SEM.

Results

Cloning and sequence analysis of tilapia CLC chloride channels

The nucleotide sequences of 2 clones from the gill cDNA library were highly homologous to rat CLC-5 and rat CLC-3. Therefore, the 3.8- and 4.2-kbp clones obtained from the gill cDNA libraries were designated as OmCLC-5 and OmCLC-3, respectively. Sequencing revealed that OmCLC-3 and OmCLC-5 were highly homologous to rat CLC-3 (72.1% nucleotide identity) and rat CLC-5 (74.1% nucleotide identity), respectively (Fig.4).

The open reading frame of OmCLC-3 is consisted of 2,280 nucleotides, resulting in a 760 amino-acid protein with a molecular mass of approximately 85 kDa. OmCLC-5 encodes an 841 amino-acid protein with a molecular mass of about 92 kDa. Fig. 5 shows hydrophathy analysis of the predicted protein of OmCLC-3 and OmCLC-5. The hydrophathy profiles show the presence of at least 12 hydrophobic regions which may represent transmembrane domains, similar to that detected in other members of the CLC family (Jentsch et al., 1995). The Asn-422 of OmCLC-3 located between hydrophobic regions D8 and D9 (Fig.4) is a highly conserved glycosylation site among all CLC chloride channels identified to date. There are also consensus sequences for phosphorylation by cAMP-dependent protein kinase (Thr-360 and Thr-361) (Kornfeld and Kornfeld, 1985) and by protein kinase C (Ser-52, Ser-363, Ser-647, Ser-693 and Thr-744) (Kemp and Pearson, 1990; Kennelly and Krebs, 1991). OmCLC-5 also has a

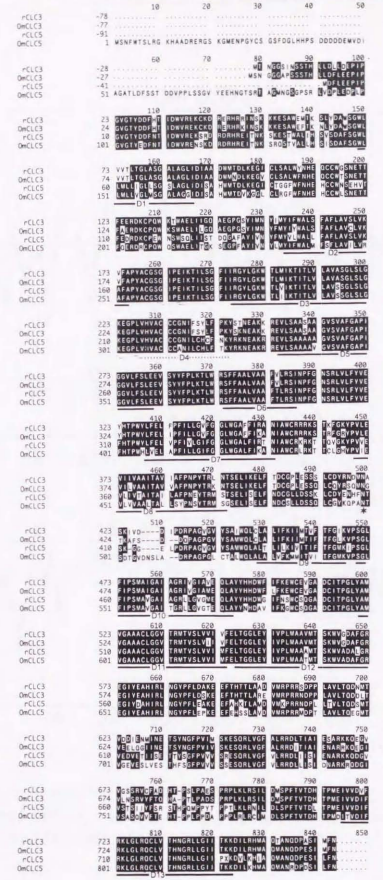


Fig. 4 Amino acid sequences of OmCLC-3 and OmCLC-5. Conserved residues are filled in black blocks and putative transmembrane-spanning domains are underlined according to the original topology by Jentsch *et al.* (1990). Potential *N*-linked glycosylation sites are indicated by asterisk.

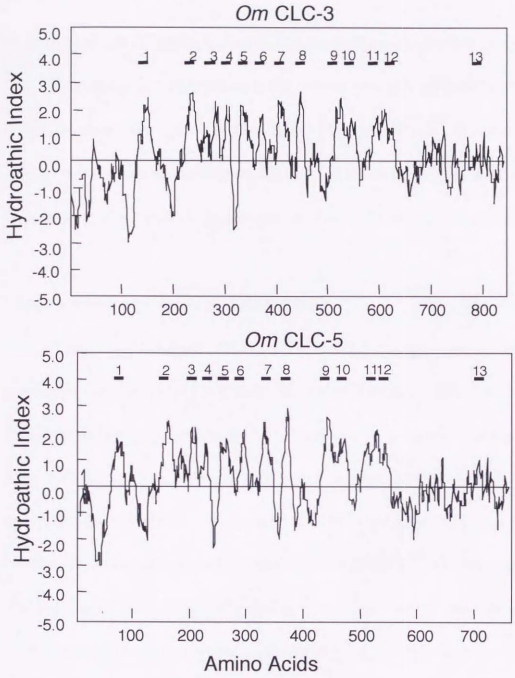


Fig. 5 Hydrophathy profile of OmCLC-3 and OmCLC-5. The mean hydrophathy index was computed according to the algorithm of Kyte and Doolittle.

potential N-glycosylation site at Asn-499 located between hydrophobic region D8 and D9. The potential phosphorylation site by protein kinase A is Thr-441, and those by protein kinase are Ser-7, Ser-724, Thr-77, Thr-128, and Thr-218. To establish a possible evolutionary relationship among the CLC chloride channels, a phylogenetic tree was constructed using DNASIS computer software (Hitachi Software Engineering) (Fig. 6).

Tissue distribution of OmCLC-3 and OmCLC-5

Tissue distributions of OmCLC-3 and OmCLC-5 were examined by RNase protection assay using total RNA from FW- and SW-adapted tilapia tissues (Fig. 7). Protected bands migrated at 150 bp. The OmCLC-3 was expressed at high levels in the brain, at moderate levels in the gill, kidney, and intestine, and at low levels in the liver, spleen, and skeletal muscle. There were no differences in the density and distribution of OmCLC-3 expression between FW and SW fish. The OmCLC-5 was expressed at high levels in the gill, kidney, and intestine. In contrast to OmCLC-3, however, the level of expression was much lower in the brain and spleen, and there was no detectable expression in the liver and skeletal muscle.

Expression of OmCLC-3 and OmCLC-5 in the osmoregulatory organs

Levels of OmCLC-3 and OmCLC-5 expression in FW and SW fish were quantitated from the RNase protection assay as the ratio of OmCLCs to β-actin mRNA. Although the expression of OmCLCs in the gill, kidney and intestine were not different

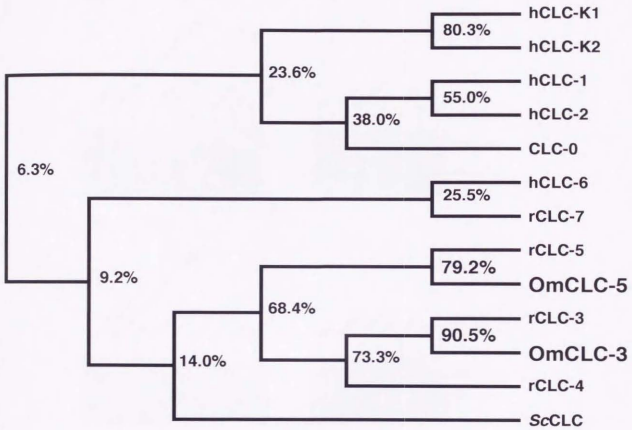


Fig. 6 Evolutionary relationship between CLC chloride channels. The phylogenic tree shows the relationship among thirteen different members of the CLC family derived from yeast, *Torpedo*, tilapia, and rat. The phylogenic tree was constructed using the DNASIS computer software (Hitachi Software Engineering).

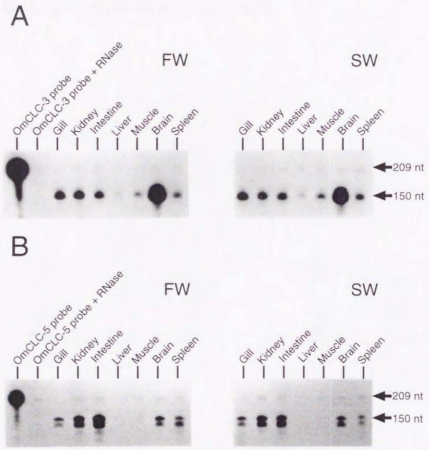


Fig. 7 Analysis of tissue distribution of OmCLC-3 (A) and OmCLC-5 (B) expression in *Oreochromis mosammbicus* by RNase protection assays. Probes were incubated with 20 mg of total RNA from yeast, and RNase protection analysis of 20 mg of total RNA isolated from gill, kidney, intestine, liver, muscle, brain and spleen of FW- and SW-adapted tilapia.

between FW and SW, the levels of OmCLC-5 expression in SW fish was constantly higher than those in FW (Fig. 8). In 2xSW-adapted tilapia, there was a significantly increase of OmCLC-5 expression in the gill (Fig.9).

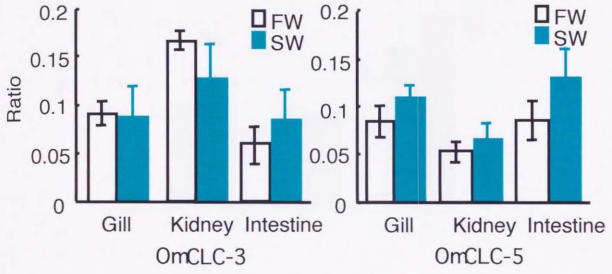


Fig. 8 Quantitative analysis of OmCLC-3 and OmCLC-5 mRNA expression in FW- and SW-adapted tilapia by RNase protection assay. The intensity of radioactivity of the protected band was measured by FUJI Bioimage Analyzer BAS-2000 (Fuji Film, Tokyo, Japan). Data were corrected by the expression rate of β-Actin. Data are expressed as the mean ± S.E.M. (n=5).

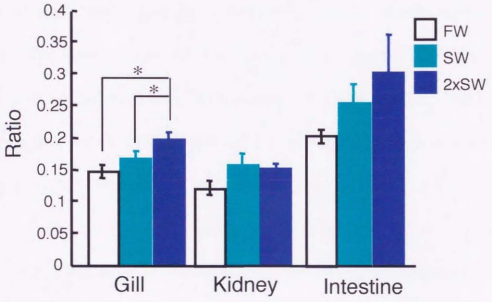


Fig. 9 OmCLC-5 mRNA expression in the gill, kidney and intestine of FW-, SW-and 2xSW-adapted tilapia by RNase protection assay. Data are expressed as the mean $\pm$ S.E.M. (n=6). *$p<0.05$ different from the values of FW- and SW-adapted fish.

Discussion

The present study described the cloning of two CLC chloride channels from the tilapia, *Oreochromis mossambicus*. The OmCLC-3 and OmCLC-5 are the first CLC chloride channels identified in the teleostean species. Among the members of CLC chloride channel family, these clones were most closely related to the mammalian CLC-3, -4 and -5 subfamily. OmCLC-3 and OmCLC-5 are highly homologous with rat CLC-3 (90.5%) and rat CLC-5 (79.2%), respectively, at the amino acid level. These results suggest that OmCLC-3 and OmCLC-5 may represent tilapia homologues of mammalian CLC-3 and CLC-5.

As is the case with the mammalian CLC-3 (Kawasaki et al., 1994), OmCLC-3 is ubiquitously expressed in all tissues examined with the highest expression in the brain. Accordingly, OmCLC-3 may have a function similar to that of the mammalian CLC-3 as a swelling-activated chloride channel (Duan et al., 1997). However, it is also possible that OmCLC-3 plays a role in acid-base regulation at the intracellular organelles rather than in the plasma membrane. Accordingly, it is less likely that OmCLC-3 is involved in cell volume regulation in tilapia. Further functional information such as cellular localization of OmCLC-3 is necessary to elucidate its functional role.

Previous studies have shown that rat CLC-5 (Sakamoto et al., 1996) and human CLC-5 (Fisher et al., 1995) are predominantly expressed in the kidney, and they are less abundantly in the brain, liver, lung, and intestine. Recently, Lindenthal *et al.* (1997)

reported that *Xenopus* CLC-5 is highly expressed in oocytes, kidney, and intestine. In the present study, OmCLC-5 is highly expressed in the gill, kidney, and intestine. These results suggest that kidney and intestine are common organs of CLC-5 expression in teleost fishes to mammals. Recently, it was reported that the dysfunctional mutation of human CLC-5 results in low molecule proteinuria (Morimoto et al., 1998). According to Gunther et al. (1998), CLC-5 colocalizes with the H^+-ATPase (V-ATPase) in proximal tubules and in intercalated cells of collecting duct of rat kidney. The intercalated cells are involved in acid-base transport. In tilapia larvae, V-ATPase immunoreactivity was present in the apical regions of pavement cells of the yolk-sac membrane in the FW-adapted larvae (Hiroi et al., 1998). In the catfish gill epithelium, Laurent et al. (1994) identified vesicles which have morphologically very similar to the proton pump vesicles found in toad urinary bladder. The vesicles are restricted to pavement cells and inserted into the apical membrane in response to changes in the acid-base balance. Considering the colocalization of CLC-5 and V-ATPase in the human kidney, OmCLC-5 could also be colocalized with the V-ATPase in the pavement cell in the gill and is related to regulate the acid-base balance in tipi.

Collectively, the isolation of two CLC genes from *Oreochromis mossambicus* will facilitate the identification of other ion transporters in teleost, and will provide an important clue to our understanding of the physiological roles of chloride channels in teleost.

Chapter 2

Functional characterization of OmCLC-3 and OmCLC-5 expressed in *Xenopus* oocytes and CLC gene-deleted yeast

Abstract

To investigate the physiological role of tilapia chloride channels, OmCLC-3 and OmCLC-5, we sought to characterize its function using expression systems in *Xenopus* oocyte and CLC-deleted yeast. Chloride currents measured by whole cell patch clamp in *Xenopus* oocytes expressing OmCLC-3 and OmCLC-5 cRNAs are both outwardly rectified. Although both expressed currents were significantly affected by chloride channel blocker DIDS, these currents were too small to demonstrate the inhibition compared with those of mammalian CLC-3 and CLC-5 (ca. 1/50-1/100). However, expression of these clones did functionally complement the gef1 phenotype of YPH250(gef), a yeast strain in which a single CLC channel (GEF1) has been disrupted by homologous recombination. These results clearly showed that OmCLC-3 and OmCLC-5 belong to the CLC chloride channel family.

Introduction

Transepithelial chloride transport has been characterized in many tissues including the gill of teleosts. Two routes have been postulated for transepithelial chloride transport, namely, transcellular and paracellular pathways. Transcellular chloride transport needs some specific transporters and/or channels for Cl^- in both apical and basolateral membranes. Numerous studies suggest a possible existence of chloride channels in the gill epithelia (Zadunaisky, 1984; Marshall, 1995); however, the molecular identity of chloride channels remains undetermined.

The CLC chloride channel family, originally established in the elasmobranch electric organ (CLC-O) (Jentsch et al., 1990), is now identified in bacteria (Fujita et al., 1994), yeast (Greene et al., 1993), plants (Lurin et al., 1996; Hechenberger et al., 1996), amphibians (Lindenthal et al., 1997), and mammals. Nine CLC genes have been discovered so far in the same mammalian species. The CLC-1 is involved in the contraction of the muscle fiber in the skeletal muscle (Steinmeyer et al., 1991), while the CLC-2 possibly plays a role in the regulation of cell volume (Thiemann et al., 1992). The CLC-K1 and CLC-K2 are exclusively expressed in the kidney and involved in transepithelial chloride transport (Uchida et al., 1993; Kieferle et al., 1994; Adachi et al., 1994). The CLC-3 (Kawasaki et al., 1994), -4 (van Slegtenhorst et al., 1994), and –5 (Fisher et al., 1995; Sakamoto et al., 1996) constitute a subfamily of this group. The CLC-3 is recently shown to be a volume-regulated chloride channel (Duan et al., 1997).

The loss-of-function mutations of human CLC-5 were shown to result in X-linked recessive nephrolithiasis (Lloyd et al., 1997).

Only one CLC homologue is present in the yeast. Its gene, GEF1, is most closely related to this subfamily. Since disruption of this yeast gene results in reduced ability to utilize glycerol and ethanol in the absence of supplemented iron, GEF1 is somehow involved in respiration and iron metabolism (Greene et al., 1993). Therefore, it is possible to identify the function of a cloned gene as a CLC chloride channel using the CLC-deleted yeast strain.

We have recently isolated two clones, OmCLC-3 and OmCLC-5, homologous to CLC chloride channels from tilapia gill. Furthermore, ribonuclease protection assay revealed that OmCLC-3 is expressed in various tissues of tilapia, while the expression of OmCLC-5 is restricted to the osmoregulatory organs such as the gill, kidney and intestine. It is likely that OmCLC-5 acts as a chloride channel and is responsible for osmoregulation of tilapia. However, it has not yet been determined whether OmCLC-3 and -5 in fact belong to the CLC chloride channel family and transport Cl^-.

Here we characterized the function of OmCLC-3 and OmCLC-5 using a whole cell clamp technique in *Xenopus* oocyte expressed with the clones. Furthermore, its function as CLC chloride channels were assessed by functional complementation of the CLC gene using a yeast strain in which a single CLC channel had been disrupted.

Materials and methods

Functional expression of OmCLC-5 and OmCLC-3 in Xenopus oocytes

The tilapia CLC-3 and CLC-5 were cloned into the pUC18 cloning vector with the SP6 promoter and *Xenopus* β-globin 5'UTR sequences. Using an *in vitro* capped RNA transcription kit (mMESSAGR mMACHINE SP6 kit, Ambion, Austin, TX, U.S.A.), capped cRNAs were transcribed from the construct after linearization. For expression studies, oocytes were defolliculated manually after collagenase digestion. Two hours after defolliculation, Stage V/VI oocytes were injected with either 20 nl of nuclease-free water or 20 nl of 1.0 ng/nl cRNA solution as prepared above. The injected oocytes were incubated in modified Barth's solution (88.0 mM NaCl, 1.0 mM KCl, 2.4 mM $NaHCO_3$, 5.0 mM Tris-HCl, 0.82 mM $MgSO_4$, 0.33 mM $Ca(NO_3)_2$, 0.41 mM $CaCl_2$, pH 7.5). After 2-3 days at 17 °C, they were mounted in a recording chamber and impaled with microelectrodes for measurement of current and voltage. Data acquisition and analysis were performed using commercially available software (Clampex pClamp5, Axon Instrument, Foster City, TX, U.S.A.). The oocytes were clamped at -30 mV holding potential, and 1 sec voltage steps from -80 to +100 mV in 20 mV increments were applied. All recordings were performed at room temperature in ND96 buffer (96 mM NaCl, 1.8 mM $CaCl_2$, 1 mM $MgCl_2$, 5 mM HEPES, pH7.4).

Generation of gef1 mutant and functional complementation of gef1 yeast mutant

The GEF1 gene disruption was introduced into the strain YPH250 (*ura3-52,*

lys2-801, ade2-101, trp1-Δ1, his3-Δ200, leu2-Δ1) (Sikorski et al., 1995). Two synthetic oligonucleotides (57 mer) were prepared for amplification of the HIS3 gene by PCR using the pRS313 vector (Sikorski et al., 1995) as a template. For homologous recombination, 40-nucleotide sequences corresponding to 750-789 base and 1835-1874 base of the GEF1 open reading frame were incorporated into these primers at the 5'-ends. Accordingly, the PCR product contained HIS3 gene flanked by 40 bp GEF1 sequences. The cells transformed with this PCR product were selected on yeast nitrogen base without histidine, yeast synthetic dropout (SD)/-His plates, and genomic DNAs from several transformants were analyzed to confirm homologous recombination by sequencing the mutated allele. The recombinant yeast phenotype was checked by growth on a YPGE (glycerol and ethanol as non-fermentable carbon source) plate, a YPGE + 1mM Ferrozine (iron-specific chelator) plate, and a YPGa (galactose, 2%) plate (Greene et al., 1993; Gaxiola et al., 1998) (Fig.10). These clones were not able to grow on agar plates containing the non-fermentable carbon sources or the Ferrozine. In our complementation assay, we could not restore the growth of gef1 mutant on the YPGE plate even by introducing wild-type GEF1 in our expression vector. Therefore, the established gef1 yeast strains YPH250(gef) were transformed with yeast expression vector pVT102-U containing OmCLC-3, OmCLC-5, or GEF1 cDNAs. OmCLC-3-, OmCLC-5-, and gef1-transformed cells were plated on YPGa agar plates. Open reading frames of OmCLC-3 and OmCLC-5 were

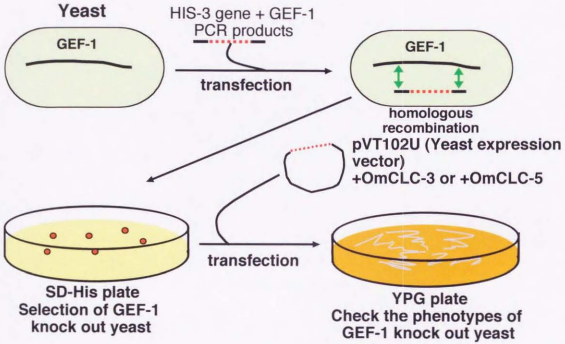


Fig. 10 Illustration of the preparation of gef1 mutant. For functional complementation analysis of gef1 mutant, OmCLC-3 and OmCLC-5-transformed yeast are incubated at 30 °C for 10 days.

subcloned into pVT102-U (Vernet et al., 1987) expression vector and transformed into several gef1 strains. Transformed cells were selected on an SD/-His/-Ura plate and yeast phenotypes were checked on YPGa plates.

Results

Functional expression of OmCLC-5 and OmCLC-3 in Xenopus oocytes

After 2-3 days of incubation of *Xenopus* oocytes injected with the cRNAs of OmCLC-3 and -5, two-electrode voltage-clamping revealed slightly outwardly rectifying chloride currents by expression of both OmCLC-3 and OmCLC-5 genes (Fig. 11). The mean currents in OmCLC-3 and OmCLC-5 injected oocytes measured at +80 mV were 251±11 nA and 80±9 nA, respectively (n=15). Addition of DIDS, specific chloride channel blocker at 500 μM reduced the current to 25±7 nA and 18±5 nA, respectively.

Functional complementation of yeast gef1 mutant by OmCLC-3 and OmCLC-5

By homologous recombination, we generated a gef1 mutant with deleted CLC whose phenotypes had been described previously (Greene et al., 1993). The phenotypes of gef1 were checked by plating on a YPGa agar plate containing 1 mM Ferrozine. We obtained four clones that exhibited a phenotype similar to the original gef1 mutants that had been obtained previously. Although gef1 mutant transformed with an empty vector could not grow on YPGa, both OmCLC-3 and OmCLC-5-transformed cells were able to grow on the plate as in the case of GEF1-transformed cells (Fig. 12). The functional complementation of the gef1 phenotype by OmCLC-3 and OmCLC-5 was also confirmed on the YPGa-Ferrozine plate (data not shown).

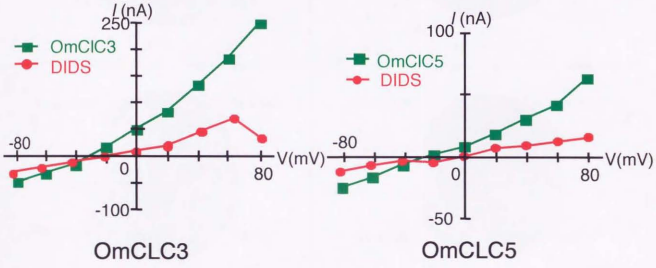


Fig. 11 Representative current-voltage relationships for oocytes injected with OmCLC-3 and OmCLC-5 cRNA. Red line indicate the effect of DIDS (500mM) application on *I-V* relationship.

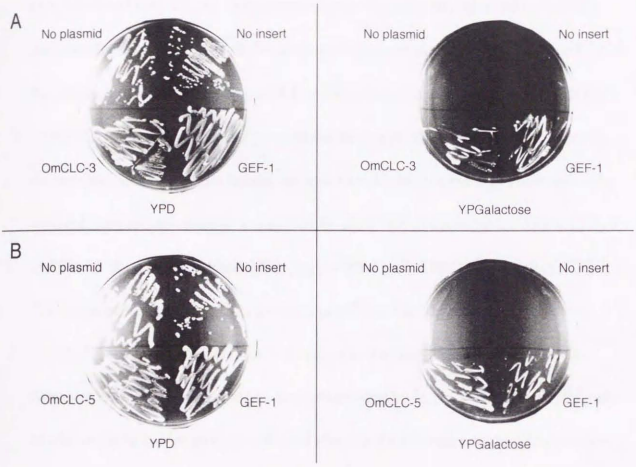


Fig. 12 Functional complementation of *gef1* yeast mutant by OmCLC-3 and OmCLC-5. YPH250(*gef*) was transformed with the expression vector containing either OmCLC-3 (A), OmCLC-5 (B), or the wild-type *GEF1* gene, or with the expression vector alone. Transformed cells and *gef1* mutant were then plated on the YPD or YPGa agar plate. OmCLC-3- and OmCLC-5-transformed cells were able to grow on the YPG plate as well as the GEF1 transformed cells.

Discussion

We were able to detect the chloride current in *Xenopus* oocyte injected with OmCLC-3 and OmCLC-5. These currents were significantly inhibited by DIDS. Although these currents have characteristics common to mammalian CLC-3 and CLC-5, the currents produced by the tilapia CLC chloride channels were much smaller (about 1/50-1/100) than those produced by mammalian counterparts. In some experiments, the nonspecific endogenous currents can also be induced in oocytes by overexpressing different integral membrane proteins (Shimbo et al. 1995, Tzounopoulos 1995), CLC-6 and CLC-7 (Brandt and Jentsch 1995), and even by a nonfunctional mutant of CLC-1 (Steinmeyer et al., 1994). Thus there is a possibility that the currents produced by OmCLC-3 and OmCLC-5 are due to the endogenous chloride channels of oocytes. However, it is also possible that low currents induced by tilapia CLC chloride channels are due to the lack of proper physiological stimulus for channel activation in the tilapia.

In our complementation assay, we could not restore the growth of gef1 mutant on the YPGE plate even by introducing wild-type GEF1 in our expression vector. This is probably due to the low promoter activity of our expression vector on the YPGE plate. Accordingly, we performed a complementation assay on the YPGa plate since the gef1 mutant could not grow on the YPGa plate, either.

As was the case with GEF1, the introduction of OmCLC-3 and OmCLC-5 into the gef1 mutant restored the growth on a YPGa plate, suggesting that OmCLC-3 and

OmCLC-5 share the function with GEF1 in the yeast. OmCLC-3 and OmCLC-5 also restored the growth on the YPGa+Ferrozine plate (data not shown), confirming the complementation of the gef1 phenotype. Since GEF1 protein is present in late- or post-Golgi vesicles in yeast cells (Gaxiola et al., 1998), the introduced OmCLC-3 and OmCLC-5 may also be localized in Golgi vesicles. However, the growth of OmCLC-3- and OmCLC-5-transformed cells appeared slower than that transformed with wild GEF1 gene, suggesting that OmCLC-3 and OmCLC-5 may not have exactly the same function or localization in the yeast cells as GEF1. Identification of cellular localization of these channels in tilapia tissues is most important to elucidate their physiological roles in osmoregulation of tilapia.

Chapter 3

Cellular and subcellular localization of OmCLC-5 chloride channels in the gills of tilapia

Abstract

The cellular localization of OmCLC-5 was investigated in the gill of tilapia, *Oreochromis mossambicus*, adapted to FW and double-strength SW (2xSW), because the mRNA expression is significantly increased in 2xSW-adapted tilapia. Using an immunohistochemical technique, we demonstrated that OmCLC-5 was localized in specific cells in the gills of FW and 2xSW fish. In comparison with the immunohistochemical localization of Na^+, K^+-ATPase, OmCLC-5 was found to be localized specifically in the chloride cells, which actively transport Na^+ and Cl^- in the gills. Electron microscopic immunocytochemistry revealed that the staining of OmCLC-5 was observed on the basolateral side of the plasma membrane of chloride cells including the tubular system. The specific localization of OmCLC-5 in the chloride cells indicates that OmCLC-5 serves as an important chloride transporter in the chloride cells.

Introduction

Teleost fishes maintain ionic composition and osmolarity of their body fluid at levels different from external media by adjusting ion and water permeability of osmoregulatory surfaces. Among members of the family Cichlidae, mozambique tilapia, *Oreochromis mossambicus*, is one of the most salinity-tolerant species with a remarkable capacity to adapt rapidly to changes in environmental salinity, e. g. from fresh water (FW) to double-strength (2x) seawater (SW). This adaptability makes the tilapia a good model for investigating the physiology of osmoregulation. The SW-adapted tilapia, like other marine teleosts, drink SW and absorb water and ions through their intestine, while they excrete excess ions through specialized mitochondria-rich "chloride" cells present in their gill epithelia.

Previously, we cloned two CLC chloride channels (OmCLC-3 and OmCLC-5) from tilapia gills. The mRNA of OmCLC-3 was widely expressed in almost all tissues examined. The OmCLC-5 is expressed mainly in the osmoregulatory organs such as the gill, kidney and intestine. These results suggest that OmCLC-5 may be important for osmoregulation of tilapia. Moreover, the OmCLC-5 possesses a function as a chloride channel as evidenced by its expression in a yeast strain devoid of CLC. As a first step to elucidate its physiological function, localization of OmCLC-5 in the Cl^- transporting gill epithelia may provide important clues to this purpose. In this study, we examined the localization of OmCLC-5 in the gill of both FW- and

2xSW-adapted tilapia using immunohistochemical techniques. Furthermore, its subcellular localization in the specific cells of gill epithelia was examined using electron microscopic immunocytochemistry.

Materials and Methods

Fishes

Tilapia, *Oreochromis mossambicus*, weighing 15-20 g, were maintained in circulating FW tanks at 25°C. To prepare 2xSW-adapted fish, some of the fish were transferred to 50 % SW for 2 days and then to 100% SW for at least 30 days. Thereafter, some of the SW-adapted fish were transferred to 150% SW for 7 days and then to 200% SW for at least 30 days.

Generation and affinity-purification of anti-OmCLC-5 serum

A specific antiserum was raised against OmCLC-5 in rabbits by Sawady Technology Co. Ltd (Tokyo, Japan). The antigen was 30 amino-acid peptide whose sequence was Gly-Gly-Ile-Asp-Ile-Ser-Ala-His-Trp-Met-Thr-Asp-Val-Lys-Gly-Gly-Leu-Cys-Leu-Arg-Gly-Phe-Trp-Phe-Asn-His-Glu-His-Cys-Cys (Fig. 13). The amino acid sequence of the synthetic peptide had low homology with the corresponding sequence of OmCLC-3, another tilapia CLC chloride channel cloned in the previous study (Miyazaki et al., 1998). The antigen was emulsified with complete Freund's adjuvant and immunization was performed in two New Zealand white rabbits. Then boost injections were made into the rabbits with peptide in incomplete Freund's adjuvant at day 14, 42 and 56 after the first injection. A specific antiserum from the rabbit with the highest titer was collected and antiserum was affinity purified using the antigen

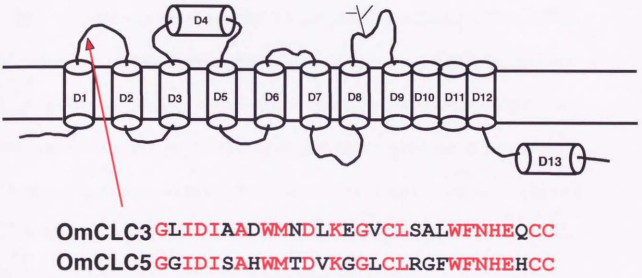


Fig. 13 The antigen of the specific antiserum for OmCLC-5. Red characters indicate conserved residues. The amino acid sequence of the synthetic peptide has low homology with the corresponding sequence of OmCLC-3 (about 63%).

attached to a cyanogen bromide-activated Sepharose column (Amersham Pharmacia Biotech, Uppsala, Sweden).

Western blotting

After anesthesia with 2-phenoxyethanol, fish were decapitated and gill arches isolated. Gill epithelia were immediately scraped off the underlying cartilage with a scalpel. Scraped cells were suspended in homogenization buffer (25 mM Tris-HCl, 0.25 M sucrose, pH7.4). Homogenization was performed in a glass Potter-Elvehjem homogenizer with a Teflon pestle at 600 rpm with 20 up-down strokes. The homogenate was then spun for 5 min at 500xg to remove nuclei and other organelles. The plasma membranes were collected by centrifugation of the supernatant for 30 min at 50,000xg. The pelletized plasma membranes were resuspended in a small volume of homogenization buffer. Protein concentration was measured, and the suspension was diluted to 2 μg protein/μl with the same buffer. Just before electrophoresis, 5 μl membrane suspension was mixed with 5 μl Tris-sodium dodecyl sulfate (SDS)-β-mercaptoethanol sample buffer (Owl Separation Systems, Inc., Woburn, MA, U.S.A.) and then heated at 95 °C for 10 min. Samples were electrophoresed on 7.5% polyacrylamide gels; kaleidoscope prestained markers (Bio-Rad, Hercules, CA, U.S.A.) were used as reference. After SDS-polyacrylamide gel electrophoresis (PAGE), proteins were electroblotted to polyvinylidene difluoride (PVDF) membrane (Hybond-P, Amersham Pharmacia Biotech, Uppsala, Sweden). After blocking the membrane

with 2% skim milk in Tris-buffered saline with 0.2% Triton-X100 for 15 h at 4 °C, the proteins were probed with an affinity-purified OmCLC-5 antibody (x400) for 1 h at room temperature. The membrane was treated with goat anti-rabbit IgG-peroxidase conjugate (Vector Laboratories, Burlingnama, CA, U.S.A.) for 2 h at room temperature, and OmCLC-5 proteins were visualized using the avidin-biotin-peroxidase (ABC) complex methods (Vector Laboratories, Burlingnama, CA, U.S.A.).

Immunohistochemistry

Tilapia adapted to different salinities were killed by decapitation, and the gills were fixed in 4% paraformaldehyde in 0.1 M phosphate buffer (PB). The gill filaments were removed, dehydrated in ethanols and embedded in Paraplast. Serial cross sections of the filaments were cut at 4 μm thickness. Pairs of adjacent sections were mounted on separate slides in a mirror-image manner; one section was stained with an anti-Na^+, K^+-ATPase serum for identification of chloride cells (Uchida et al., in press) and the other section was stained with an anti-OmCLC-5 serum. The sections were immunohistochemically visualized by the ABC method.

Electron-microscope observation of OmCLC-5 immunoreactive cells

The gills of FW-, SW- and 2xSW-adapted tilapia were fixed in 4% paraformaldehyde in 0.1 M PB, pH 7.4, for 12 h. After fixation, small pieces of the second gill arches were cut off, and whole-mount immunocytochemistry, with the ABC method, was carried out based on the method of Shiraishi et al. (1997). The gill

filaments were incubated with OmCLC-5 antibody diluted 1:400 with 0.01 M PBS containing 0.05% Triton X-100, 10% normal goat serum, 0.1% bovine serum albumin and 0.01% sodium azide (TPBS) at 4 °C overnight. The tissues were then post-fixed in 1% osmium tetroxide in TPBS for 1 hour. Subsequently, the samples were incubated with biotinylated anti-rabbit IgG (Vector Laboratories, Burlingnama, CA, U.S.A.) for overnight, followed by incubation with ABC reagent overnight. Then, the samples were washed with 0.1 M PB and soaked in 0.02% 3, 3'-diaminobenzidine tetrahydrochloride containing 0.005% H_2O_2 for 60 min. After dehydration in ethanols, the tissues were transferred to propylene oxide and embedded in Spurr's resin. Ultrathin sections were cut with a diamond knife and mounted on grids. The sections were examined with an H-700 electron microscope (Hitachi, Tokyo, Japan).

Results

Characterization of antibody

As shown by Western blotting of the proteins from the tilapia gills, OmCLC-5 antibody (OC5A) bound a single peptide of about 90 kDa (Fig. 14). The molecular mass of OmCLC-5 predicted from the cDNA sequence is 92 kDa. Pretreatment of the antibody with the antigen peptide completely abolished the band, indicating that OC5A specifically recognized the OmCLC-5 protein. Therefore, we used the OC5A for subsequent immunohistochemistry of the gills.

Immunohistochemistry of the gill

The immunohistochemical staining with the OC5A at a light microscopic level showed that OmCLC-5 immunoreactive cells were located in the filament of gill epithelia of FW- and 2xSW-adapted tilapia (Fig. 15). The number of immunoreative cells increased and the intensity of immunoreaction also increased in 2xSW-adapted fish compared with the FW fish. In adjacent sections, immunoreaction with Na^+, K^+-ATPase was observed on the same cells which were immunoreactive with OmCLC-5 although the latter exhibited much weaker immunoreactivity (Fig. 15). These results show that the OmCLC-5-immunoreactive cells were ion-transporting chloride cells.

Electronmicroscope observation of OmCLC-5 immunoreactive cells

Ultrastructural observation of OmCLC-5-immunoreactive cells of FW- and

Fig. 14 Western blotting of the 20 μg membrane fractionated protein of the tilapia gill with OmCLC-5 specific antibody (OC5A) (A). Pretreatment of the antibody with the antigen peptide (B) completely abolished the band. Molecular mass was referred by kaleidoscope prestained markers (Bio-Rad, Hercules, CA, USA).

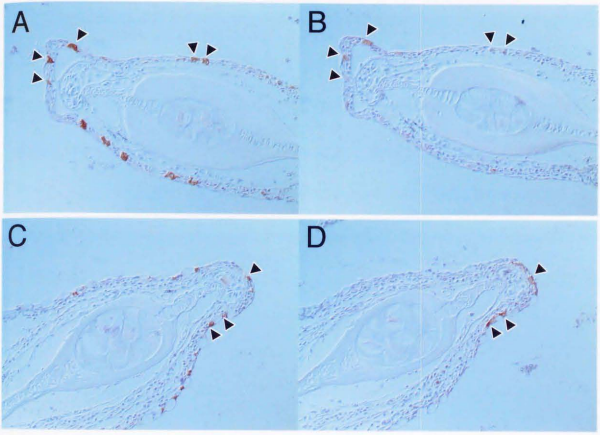


Fig. 15 The serial sections of the gills, stained with Na^+, K^+-ATPase antibody (A, C) and anti-OmCLC-5 OC5A antibody (B, D) in 2xSW- (A, B) and FW-adapted tilapia (C, D). Arrowhead indicates the cells that were stained with antibody of both Na^+, K^+-ATPase and OmCLC-5.

2xSW-adapted fish revealed that these cells were characterized by a rich population of mitochondria and an extensive labyrinth of basolateral membrane (tubular system) (Fig. 16). Immunoreaction of OmCLC-5 was also recognizable on the basolateral membrane (Fig. 16). In FW-adapted fish, OmCLC-5-immunoreactive cells contained moderately developed tubular system and a weak immunoreactive signal. In SW- and 2xSW-adapted fish, the cells were larger, the tubular network was denser, and the immunoreactive signals were stronger than those of FW fish. In comparison with the localization of Na^+, K^+-ATPase, the immunoreactions of OmCLC-5 were observed in the same positions (tubular system).

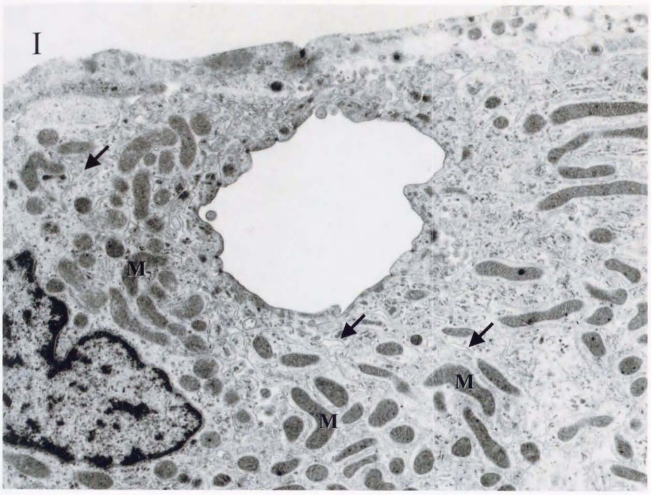


Fig. 16 (I) Transmission electron micrographs of chloride cells in the gill of 2xSW-adapted tilapia. Chloride cell are characterized by a rich population of mitochondria and an extensive basolateral membrane (tubular system).

Magnification, x8,000.

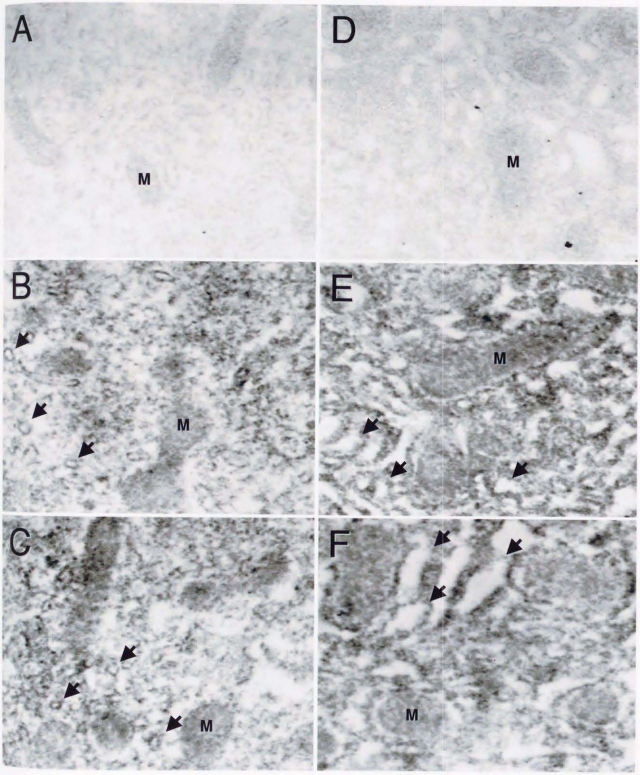
(II) Immunoelectron micrographs of filament chloride cells in tilapia adapted to FW (A, B, C) and 2xSW (D, E, F). These sections were stained with normal rabbit serum (A, D), Na^+, K^+-ATPase antibody (B, E) and OmCLC-5 antibody (C, F).

Magnification, x20,000.

M: mitochondria

Arrow: typical tubular system

II



Discussion

In the present study, we showed that OmCLC-5 is abundantly expressed in the chloride cells of FW and 2xSW tilapia as evidenced by the co-localization with Na^+, K^+-ATPase. The chloride cells are important in maintaining ionic balance in fish, being the primary site of secretion of excess Na^+ and Cl^- in SW and being the site of ion uptake to compensate the passive loss of Na^+ and Cl^- in FW. This location suggests an important role of OmCLC-5 in osmoregulatory function in tilapia.

We demonstrated, using an electron microscopic whole-mount immunocytochemistry, that OmCLC-5 is expressed at the basolateral membrane of the chloride cells in both FW- and SW-adapted tilapia. As shown in Fig. 1, models are proposed that predict differences in the composition and distribution of ion channels and transporters in the gill chloride cells between FW- and SW-adapted fish (Marshall, 1995). The driving force for Cl^- secretion in SW-adapted fish is the Na^+ gradient established by the Na^+, K^+-ATPase located in the basolateral membrane. Cl^- enters the cell across the basolateral membrane via Na^+-dependent Na^+, K^+, 2 Cl^- cotransporter. Cl^- accumulates intracellularly so that Cl^- exit occurs down its electrochemical gradient through anion (chloride) channels in the apical membrane. In the model of FW-adapted fish, the transport is also dependent on a basolaterally located Na^+, K^+-ATPase. The major step for Cl^- transport is at the apical membrane, which may be driven by Cl^-/HCO_3^- exchanger. Cl^- accumulates intracellularly, so that Cl^- exit occurs down its

electrochemical gradient through chloride channels in the basolateral membrane. Previously, a likely candidate for the basolateral chloride channel was suggested in the basolateral membrane of teleost urinary bladder using an electro-physiological technique (Chang and Loretz, 1991), but its molecular identity has not yet been determined. Therefore, OmCLC-5 is the first basolateral membrane chloride channel identified as a molecule in fish.

Recently, there is some debate on the site of Cl^- and Na^+ uptake by the gill of FW fish (e.g., Lin and Randall, 1995; Sullivan, et al., 1996; Perry, 1997; Goss et al., 1998). It is generally accepted that Cl^- uptake is achieved via the chloride cell because morphological changes in the number and structure of chloride cells correlate well with the rates of Cl^- uptake (e.g., Perry and Laurent, 1989; Goss et al., 1994). The opercular skin of FW-adapted *Fundulus heteroclitus*, which has high density of chloride cells, also exhibits net uptake of Cl^- when exposed to Ringer solution on both sides (Wood and Marshall, 1994; Marshall et al., 1997). In addition, using an X-ray microanalysis, it was determined that injection of acetazolamide or exposure to thiocyanate (both are presumed to inhibit apical Cl^-/HCO_3^- exchanger) lowered the Cl^- content of chloride cells, but not pavement cells, in the brown trout (Morgan et al., 1994; Morgan and Potts, 1995). These ion transport models of the chloride cell in FW and SW fish expect that the transport direction of Cl^- is determined by the cellular location of the chloride channels. Thus, ion-regulating mechanisms are controlled by the regulation of a

number of transporters located both apical and basolateral membranes including chloride channels.

Recently *Xenopus* CLC-5 was cloned from the renal cell line A6 (Lindenthal et al., 1997). The cultured renal epithelial cell line A6 forms well differentiated monolayers that have properties similar to the epithelia of renal distal tubule. Several types of chloride channels that have been implicated in either transepithelial Cl^- transport or cellular homeostasis have been described in the apical and basolateral membranes of polarized A6 monolayers. *Xenopus* CLC-5 have properties similar to the basolateral A6 chloride channel described by Banderali and Ehrenfeld (1996). Borchiero et al. (1995) showed that basolateral chloride channel of A6 cell plays a role in transepithelial Cl^- absorption and cell volume regulation in the hyperosmotic media. Thus, the localization of OmCLC-5 in the basolateral plasma membrane of the chloride cells indicates that the OmCLC-5 may play an important role in Cl^- absorption by the chloride cells of FW-adapted tilapia.

Recently, Zadunaisky *et al.* (1995) found that transfer of the euryhaline fish (*Fundulus heteroclitus*) from low salt water to SW involves a rapid signal that induces ion secretion by the gill chloride cell. An increase of 65 mOsm in plasma osmolarity was found during the transition. The isolated opercular epithelium of SW-adapted *Fundulus* rich in chloride cells exposed to 50 mOsm mannitol on the basolateral side show 100% increase in chloride secretion. These results show that plasma osmolarity

directly affects the transport of Cl^- in the chloride cells. In A6 cells, *Xenopus* CLC-5 plays a role in transepithelial Cl^- transport and volume regulation in hypertonic media (Brochiero et al., 1995; Banderali and Ehrenfeld, 1996). Thus, presence of OmCLC-5 in the basolateral membrane of chloride cells in SW- and 2xSW-adapted tilapia may be preparation for abrupt changes in environmental salinity.

Osmoregulatory mechanisms of euryhaline fishes are known to be under endocrine control; prolactin is a predominant osmoregulatory hormone for FW adaptation, and cortisol serves as a hormone for SW adaptation. In euryhaline fishes, these hormones seem to play a preparatory role and trigger the operation of the osmoregulatory systems to the appropriate direction since their surge in serum levels precedes FW and SW entry. In contrast, OmCLC-5 appears to be located downstream of these hormonal actions and plays a role in the long-term survival in FW and SW. Future studies should reveal how environmental hypertonicity is recognized and how the recognition is converted to the activation of the OmCLC-5 gene and protein. Furthermore, the physiological function of OmCLC-5 should be examined in association with other channels and transporters.

General Discussion and Conclusions

The classic model proposed by H. W. Smith (1932), which is substantiated by A. Krogh (1939), summarizes our knowledge of how osmoregulatory organs such as the gill, gut and kidney, work in concert to maintain mineral and water balance in aquatic animals. In FW teleosts, the passive loss of these electrolytes along the concentration gradient across the body surfaces are compensated by an active uptake of ions by the gills. In marine teleosts, SW is swallowed, and subsequent Na^+ and Cl^- entry across the gut is balanced by the active excretion of these ions by the gill.

The vast majority of fish species can only tolerate salinities of either hyper- or hypo-osmotic to their plasma, that is, FW or SW. However, there are a significant number of species named euryhaline fish that possess physiological mechanisms which enable them to survive over a wide range of salinities. One of the best-studied examples of the euryhaline species is the family Cichlidae. Among members of the family that are generally referred to as tilapia, *Oreochromis mossambicus* is one of the most salinity-tolerant species, reproducing both in FW and in SW (Stickney 1986; Suresh and Lin 1992). Furthermore, direct transfer of fertilized eggs, developing embryos, or newly hatched larvae from FW to SW or *vice versa* is possible without mortality (Kwaye et al. 1992; Ayson et al. 1994). Thus, FW- and SW-adapted juvenile and adult fishes are readily available for experiments, providing a suitable model for studies of osmoregulatory mechanisms during their whole life stages.

To clarify the mechanisms of excellent SW adaptability in tilapia and the role of the gill in this excellent mechanism, the author examined the developmental changes of Cl^- turnover rate in tilapia embryos and larvae in my master course thesis. The turnover rates of Cl^- increased markedly at hatching both in FW and SW tilapia (from 0.001 h^{-1} to 0.015 h^{-1} in FW and from 0.18 h^{-1} to 0.78 h^{-1} in SW). Similarly, the Cl^- turnover rate increased at hatching in *Fundulus heteroclitus* and *F. berumudae* in SW (from 0.026 h^{-1} to 2.0 h^{-1}) (Guggino 1980). The increased Cl^- turnover may be in part due to an increase in the surface area. At the time of hatching, owing to the formation of the adult gill structure including filaments and lamellae, there are large increases in surface area of the body. Gray (1954) has shown that the gill of adult teleosts occupies most of the surface area of the whole body. He found that in 17 species of teleosts the gill surface area alone accounts for 1.3-18.3 times the remaining surface area of the body. Therefore, the increase in the turnover rate of Cl^- at hatching might be attributed to the development of the gill, resulting in an increase in the surface-to-volume ratio. Therefore, the gill is the most important organ that is concerned in ion exchange between body fluid and external environment. However, the fact that Cl^- turnover rate increased about 4 times at hatching in SW and 15 times in FW is difficult to be explained only by an increase in newly formed gill surfaces. Furthermore, the Cl^- turnover rates of tilapia embryos and larvae in SW were markedly higher than those in FW. It is highly probable therefore that the physico-chemical properties of the gill

epithelia in FW larvae are different from those in SW larvae. These results clearly indicate that the gills are the most important routes of Cl^- transport, and the gill epithelia have different characters between FW- and SW-adapted fish.

Since the classic observation of Krogh (1939), it has been known that in teleosts the branchial epithelium is the major site of influx and efflux of Na^+ and Cl^-. The alternation of two pumping activities oriented either outward or inward of the organism makes the branchial epithelium one of the most fascinating biological epithelial membranes. The branchial epithelium consists of a mosaic of respiratory cells and less numerous mucous cells and chloride cells, the latters being located especially at the base of the secondary lamellae (Pisam and Rambourg, 1991). In FW, passive losses of Na^+ and Cl^- in the body surfaces as a result of diffusion along the electrochemical gradient are compensated by active absorption of these ions through the gills. The Na^+ and Cl^- exchanges between the fish and the external medium bring about a renewal of these electrolytes in the body. The rate of this renewal (turnover rate), which can be measured by means of radioactive isotopes, is very low: about 0.2% of the internal Na^+ and Cl^- per hour (Motais and Garcia Romeu, 1972). In SW, however, the turnover rate of Na^+ and Cl^- is more than 100%.

The difference in ion permeability between FW- and SW-adapted tilapia shown in this study may be related in part to the changes in the chloride cell morphology and function. Several models have been proposed for the mechanisms of

active ion transport across the gill epithelium, including specific routes through chloride cells in fish adapted to either FW or SW (Towle 1990; Lin and Randall 1995; Marshall 1995). The driving force for Cl^- secretion in SW-adapted fish is the Na^+ gradient established by the Na^+, K^+-ATPase located in the basolateral membrane. Cl^- enters the cell across the basolateral membrane through Na^+-dependent Na^+, K^+, $2Cl^-$ cotransporter. Cl^- accumulates intracellularly such that Cl^- exit occurs down its electrochemical gradient through anion (chloride) channels in the apical membrane because intracellular potential of chloride cells is reported to be approximately -60 mV (Marshall, 1995). Na^+ secretion occurs down its electrochemical gradient via a cation-selective paracellular pathway that is located between chloride cells and the smaller adjacent (or accessory) cells.

The model in FW-adapted fish includes electrically silent anion and cation exchangers that allow uptake of Na^+ and Cl^- in roughly equivalent amounts with concomitant excretion of acid or base equivalents depending on the relative speed of operation of the two exchangers. Classically, it is assumed that the Na^+/H^+ exchangers also exchange Na^+ and NH_4^+ when ammonia is present in the cell. All cation transports are dependent on basolaterally located Na^+, K^+-ATPases. The active step for Cl^- transport is at the apical membrane, which may be driven by Cl^-/HCO_3^- exchanger. Cl^- and Na^+ accumulates intracellularly such that Cl^- and Na^+ exit occur down its electrochemical gradient through Cl^- and Na^+ channels in the basolateral

membrane. The transport routes of Cl^- were intracellulary both in FW- and SW-adapted fishes, since the cellular localization of Cl^- transporters (chloride channel and co-transporter) determined the direction for Cl^- transport. Therefore, the chloride channel is one of the key proteins for the Cl^- metabolism in fishes.

We have cloned two chloride channels (OmCLC-3 and OmCLC-5) from the gills of tilapia adapted to FW and SW. Chloride channels are passive anion transport proteins involved in basic functions common to all cells such as regulation of cell volume and intravesicular pH. In mammals, chloride channels are important for the transepithelial chloride transport in osmoregulatory organs and the regulation of excitability of muscle and nerve. The CLC chloride channel family is made up of 9 members of proteins in mammals, and identified in bacteria (Fujita et al., 1994), yeast (Greene et al., 1993), plants (Lurin et al., 1996; Hechenberger et al., 1996), elasmobranchs (Jentsch et al., 1990) and amphibians (Lindenthal et al., 1997). The CLC-3, -4, and -5 constitute a subfamily of this gene family (Kawasaki et al., 1994; van Slegtenhorst et al., 1994; Fisher et al., 1995; Sakamoto et al., 1996). The CLC-3, which was cloned in this study, is recently shown to be a volume-regulated chloride channel and was found ubiquitously in almost all cells (Duan et al., 1997). The function of CLC-5, which was also cloned in this study, has not been established, but loss-of-function mutations of human CLC-5 resulted in X-linked recessive nephrolithiasis (Lloyd et al., 1997). The OmCLC-3 and OmCLC-5 are the first CLC chloride channels

identified in teleost species. The OmCLC-3 and OmCLC-5 are, respectively, highly homologous with rat CLC-3 (90.5 % amino acid identity) and rat CLC-5 (79.2 % amino acid identity). These results suggest that OmCLC-3 and OmCLC-5 may represent homologues of CLC-3 and CLC-5 in tilapia. As is the case in mammalian CLC-3 (Kawasaki et al., 1994), OmCLC-3 is widely expressed in many tissues. Accordingly, OmCLC-3 may have a function similar to that of mammalian CLC-3 as a swelling-activated chloride channel (Duan et al., 1997). On the other hand, rat and human CLC-5 are predominantly expressed in the kidney (Sakamoto et al., 1996; Fisher, 1995). Recently, Lindenthal et al. (1997) reported that *Xenopus* CLC-5 is highly expressed in kidney and intestine. In the present study, OmCLC-5 is also abundant in osmoregulatory organ such as the gill, kidney, and intestine, particularly in the chloride cells of the gills. Therefore, CLC-5 homologues of these animals may be concerned in water and ion regulation.

The increases in OmCLC-5 expression in 2xSW-adapted fish strongly suggest the role of this protein in SW adaptation and osmoregulation. The mechanism by which OmCLC-5 gene expression increases during SW adaptation is not known; however, this could be a result of transcriptional activation or a reduction in mRNA turnover. Further investigation as to the role of OmCLC-5 in adaptation to salinity changes will lead to an understanding of the molecular mechanisms of the teleost osmoregulation as well as deepen our understanding of the OmCLC-5 function.

The high expression of OmCLC-5 in the gill of 2xSW-adapted tilapia is in accord with the fact that the gills abundantly contain specialized Cl^- secreting chloride cells. Using the vibrating probe technique, Foskett and Scheffey (1982) demonstrated that, in SW-adapted tilapia, the Cl^- movement is localized to the chloride cells. The co-localization of OmCLC-5 and Na^+, K^+-ATPase shown in this study demonstrates that OmCLC-5 is localized in the chloride cells of tilapia. The chloride cells are important in maintaining ionic balance in fish, being the primary site of secretion of excess Na^+ and Cl^- in SW and the site of uptake of Na^+ and Cl^- in FW. Since chloride cells are rich in Na^+, K^+-ATPase reflecting their extraordinary power of ion transport, OmCLC-5 in the chloride cells may be an osmoregulatory component working cooperatively with this enzyme. Since OmCLC-5 protein is expressed in both FW- and SW-adapted tilapia, it may performs important functions in both excretion and absorption of Cl^-.

We demonstrated using electron microscopic (EM) immunohistochemistry that the OmCLC-5 is localized on the basolateral membrane of the chloride cells. This localization indicates that OmCLC-5 may play an important role in Cl^- absorption by the chloride cells of FW-adapted tilapia. While we are not able to detect OmCLC-5 in the apical membrane as shown in Chapter 2, the increment of mRNA levels of OmCLC-5 in the gill of 2xSW-adapted fish indicates that OmCLC-5 is related to hypoosmoregulation.

During the course of SW adaptation, the number of chloride cells increases and the intercellular junctions between the cells get looser as evidenced by the increased permeation of La^{3+} (Sardet et al., 1979). This structure is usually called "paracellular pathway" or "leaky junction". This "hyperosmotic response" is accompanied by hypertrophy of the preexisting chloride cells and amplification of the tubular system (Philpott, 1980). At the same time, an increase in ion exchange occurs. Exchanges of Na^+ and Cl^- across the gills increase so that 25-60% of the total Cl^- content of the fish is exchanged per hour in SW. Moreover, Epstein et al. (1967) found that the acclimation of *Fundulus heteroclitus* to SW is associated with a profound increase in branchial Na^+, K^+-ATPase. The Na^+, K^+-ATPase that is actually localized on the basolateral membrane of chloride cells actively transports Na^+ to the "paracellular pathway". Therefore, the "paracellular pathway" is very high in Na^+ concentration and is also very positive in electric potential. We demonstrated using EM immunohistochemistry that the OmCLC-5 is localized on the basolateral membrane of the chloride cells and co-localized with Na^+, K^+-ATPase. Thus, intracellular Cl^- is moved to "paracellular pathway" by its electrochemical gradient. Supposing that these ions are continually dumped into the long and narrow "paracellular pathway" by the active ion pump and OmCLC-5, this processes make this space hypertonic to the environments. Then the ions may continually diffuse down its concentration gradient toward environment through the opening of the "leaky junction" (Fig. 17).

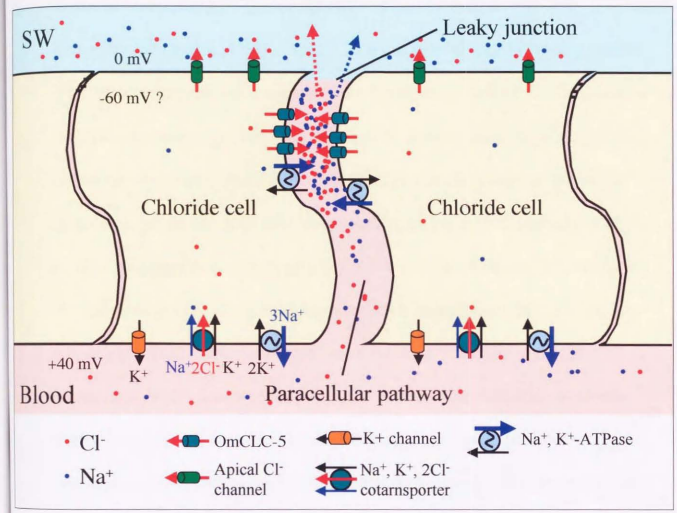


Fig. 17 A new model for ion excretion in the chloride cells of SW-adapted fish. Co-localization of Na^+, K^+-ATPase and OmCLC-5 generates very high concentration of Na^+ and Cl^- in the "paracellular pathway" that is higher than SW. Thereafter, these ions may continually diffuse down its concentration gradient toward the opening of the "leaky junction". Passive diffusion of Na^+ and Cl^- is indicated by dashed lines.

Recently, another family of chloride channel, cystic fibrosis transmembrane conductance regulator (CFTR), was cloned from the gill of killifish, *Fundulus heteroclitus* (Singer et al., 1998). The CFTR is a member of the ATP-binding cassette (ABC) transporter gene family and a cAMP-regulated chloride channel. The mutation in CFTR gene causes cystic fibrosis (CF), one of the most common genetic lung diseases in Caucasians. Patch clamp studies in the epithelial cells of patient with CF have shown that the cAMP-dependent outwardly rectifying chloride channel (ORCC) is not properly regulated in these patients (Welsh et al., 1986; Frizzell et al., 1986; Li et al., 1988; Hwang et al., 1989), indicating the possible importance of ORCC in the pathogenesis of CF. When the CFTR gene was cloned (Rommens et al., 1989; Riordan et al., 1989), it soon became clear that CFTR encodes a small linear chloride channel and not ORCC. Although, this discrepancy has been puzzling the investigators, recent findings that CFTR acts as an ATP-permeating channel may solve this problem. Schwiebert *et al.* (1995) have shown that CFTR is able to transport ATP outwardly in addition to Cl^- and that ATP transported from outside the cell binds ATP receptor and stimulates ORCC. Based on these findings, attention has been focused on the molecular identity of ORCC. Sakamoto et al. (1995) reported that rat CLC-5 has a character of outwardly rectifying chloride channel and can be activatable by CFTR. In the present study, we observed that OmCLC-5 expressed in *Xenopus* oocyte exhibited only a weak outwardly rectifying chloride conductance. In SW-

adapted killifish, CFTR was expressed at high levels in the gill, as OmCLC-5 shown in this thesis. Thus, OmCLC-5 might be one of the candidates of ORCC that is controlled by CFTR also in teleost.

Osmoregulatory mechanisms of euryhaline fishes are known to be under endocrine control. The cortisol, growth hormone (GH), insulin-like growth factor I (IGF-I), and thyroid hormones (T_3 and T_4) increase Na^+, K^+-ATPase and promote the differentiation of chloride cells (McCormick, 1995). Prolactin inhibits the development of chloride cells and promotes the differentiation to FW type chloride cell. Recently, cortisol, GH and prolactin receptors are cloned from some teleost species, and they are shown to be expressed in the chloride cells (Sandra et al., 1995; Uchida et al., 1998). Although the direct action of these hormones on ion transport in the chloride cells has yet to be demonstrated, functional differentiation of chloride cells is presumably controlled by these hormones. Further exploration for the hormonal control of ion channels and transporters are needed, and this will be an interesting area for future investigation.

The OmCLC and isolation of the other ion transport proteins of chloride cells will be powerful tools for determining the functional attributes of chloride cells. Until now, Na^+, K^+-ATPase, H^+-ATPase, Cl^- channels (i.e. OmCLC and kfCFTR), K^+ channel, Cl^-/HCO_3^- exchanger and Na^+, K^+, $2Cl^-$cotransporter have been cloned from fish. However, their physiological properties have been discussed only separately at

individual protein. Therefore, further exploration of the functional cooperation of these transporters and hormone receptors in the chloride cells are needed. Continued development of the methods that allow co-expression systems of these transporters and hormone receptors will promote our understanding of direct interactions. Combination of the new technology with classical physiology, histology and endocrinology will be especially useful to deepen our understanding of ion transport mechanisms in the chloride cells.

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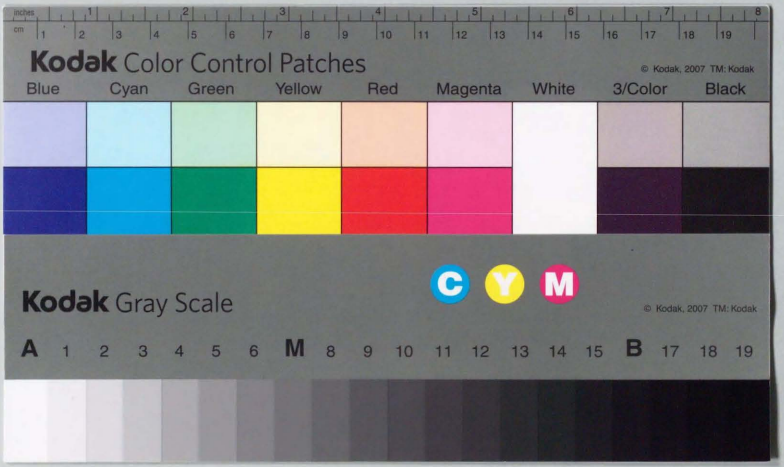
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