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Cloning, properties, and localization of an RBCC protein specifically expressed in freshwater eel gill

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ABBREVIATIONS

GST	glutathione *S*-transferase
RBCC	RING finger, B- box, and coiled-coil
HEPES	*N*-2-hydroxyethylpiperazine-*N*'-2-ethanesulfonic acid
EDTA	ethylenediaminetetraacetic acid
TPEN	tetrakis-(2-pyridylmethyl) ethylenediamine
Ub	ubiquitin

GENERAL INTRODUCTION

About 70% of the body weight of animals is water. In the water inside and outside of the cells, a variety of solutes are dissolved, and the maintenance of the composition of each of these solutions is necessary to vital for life. The suitable compositions of these solutions provide an inner environment with electrolyte content and an osmolality suitable for life. And the osmoregulation is the one of the most important factor for all animal life.

Fishes live in water environment and respire with the aid of gills. The gill is permeable organ, so the difference between the salt concentrations of the environmental solution and their body fluid causes the osmotic diffusional movement of water and salt in the gills. As a result, fishes must maintain continuously osmolality with their osmoregulation organs (Fig. 1). In the freshwater, the environmental solution is hypoosmotic to their body fluids. Therefore there is a tendency to gain water and lose salts across the gill membrane. So fishes must excrete water and uptake the salts.

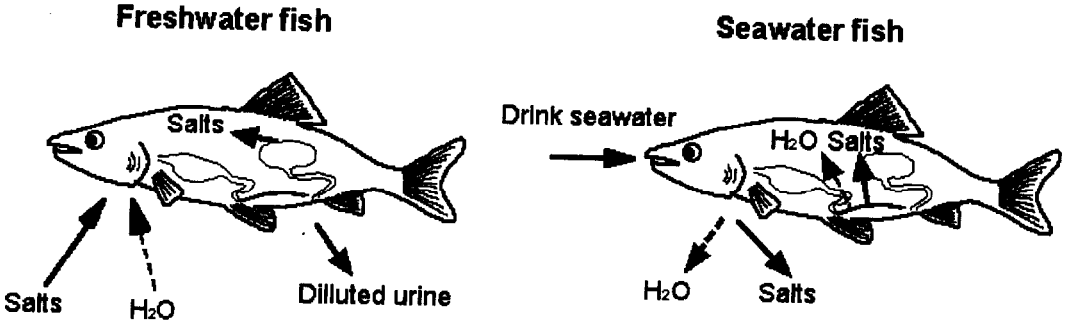

Fig. 1. Osmoregulation of the fish in the freshwater and seawater

Though salts are principally obtained from the food, additional gains of sodium chloride may be made as a result of its active transport, against electrochemical gradients, across the gills. It has been suggested that the fishes in the freshwater produce a dilute urine in the kidney and uptake salts from the gill to maintain their body fluids. On the other hand, in the seawater, the environmental solution is hyperosmotic to their body fluids. Therefore there is a tendency to lose water and gain salts across the gill membrane. It is necessary for the maintenance of their body fluids to uptake water and excrete salts. So marine fishes drink seawater. Much of the salt in seawater is absorbed across the gut wall, and water follows this solute by osmosis. The salt is excreted by special cells in the gill. The special cells are called chloride cell.

Chloride cells are localized in gill epithelium. They are characterized by numerous large mitochondria and a complex microtubular system. And The basolateral membranes of them have high concentrations of Na^+,K^+-ATPase [1,2]. The chloride cells were considered to be the site for responsible for excretion of excess sodium and chloride in the body fluid of seawater fish, and their proliferation and differentiation were induced in fish placed in seawater [3]. However, now two types of chloride cells, seawater type and freshwater type, have been known in euryhaline fish [4]. The seawater type chloride cells are detected in filament epithelia of gills, and the freshwater type chloride cells are detected in lamella epithelia of gills (Fig. 2). The finding of the two types of chloride cells suggests an important role of the chloride cell in both environments, freshwater and seawater.

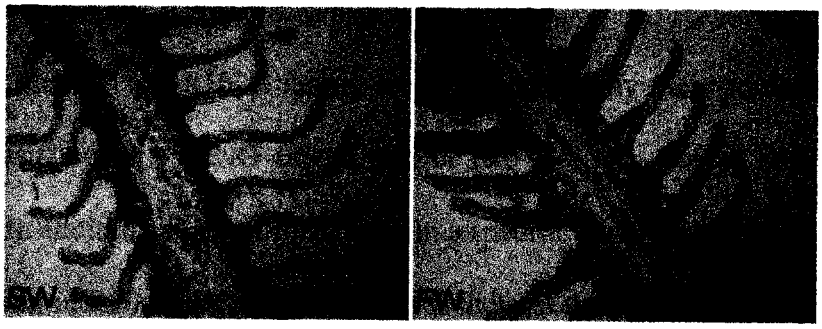

Fig. 2. Distribution of chloride cells in the seawater and freshwater gill. mRNA of Na^+,K^+-ATPase, the marker gene of chloride cell, was detected in seawater or freshwater-adapted eel gill by *in situ* hybridization. Scale bar represents 25 μm. SW, seawater; FW, freshwater

The euryhaline fishes can migrate between freshwater and seawater because of their osmoregulation mechanism. So they are suitable for the studies of osmoregulation. Now the studies of proliferation and differentiation in the chloride cells have done from the endocrinological, physiological, and morphological point of view (Table1). But the studies at the molecular level are very few especially in freshwater adaptation. And the protein that works as a regulator of proliferation and differentiation has not found yet. So I initiated this study, identification of the regulatory molecules in the gill of eel, the euryhaline fish, to clarify the osmoregulation in the freshwater at the molecular level.

Differential display is the method by which the difference of mRNA expression between two samples is identified. I used this method and tried to identify the mRNA expressed much more highly in the gill of freshwater eel than seawater eel. As such a protein, I found a novel protein belonging to the RBCC (RING finger–B-box–coiled-coil) family that is induced in the freshwater eel gill. I named the protein

"eRBCC" (e for eel) and characterized it.

Members of RBCC family are characterized by their possession of a tripatic motif consisting of a RING finger, one or two B-boxes and an α-helical coiled-coil domain. In addition, some of them contain a specific carboxy-terminal region known as RFP domain or B30.2-like domain [5]. It is thought that the domains composing RBCC are involved in protein-protein interactions and allow members of RBCC family to participate in various cellular phenomena, such as differentiation, development, oncogenesis, and apoptosis, depending on their subcellular localization. And some of the members of RBCC family are known that have the ubiquitin ligase (E3) activity and are involved in proteolysis [6]. In this study, I identified eRBCC as a gene involved in osmoregulation adapting in freshwater. eRBCC is localized in the nucleus of the gill epithelial cells containing chloride cells and has the ubiquitin ligase (E3) activity. This finding suggests that eRBCC is one of the regulator genes of the cell proliferation or differentiation in the osmoregulation in freshwater adaptation.

Table 1. Osmoregulatory response to hormones in teleosts

Target organ	Stimulatory hormone	Nature of response	Phyletic distribution of responsiveness
Kidney	Vasotocin	Decreased GFR: antidiuresis	Eel
		Increased GFR: diuresis	Some teleosts and lungfishes
Urinary bladder	Prolactin	Reduced water permeable, increase Na^+ reabsorption	Starry flounder
	Urotensin II	Increase Na^+ reabsorption	Goby
Gills	Cortisol	Increase outward Na^+ secretion	Marine teleosts
		Increase inward Na^+ absorption	Freshwater teleosts
	Growth hormone	Proliferation of chloride cells	Teleosts
	Natriuretic peptides	Increase Na^+ efflux	Teleosts (SW)
	Prolactin	Decrease Na^+ diffusion outward and water accumulation	Euryhaline teleosts (FW)
		Decrease Na^+ extrusion	Euryhaline teleosts (SW)
	Urotensin I	Increase Cl^- secretion	Teleost (SW) chloride cells
	Urotensin II	Decrease Cl^- secretion	Teleost chloride cells
	Vasotocin	Increase inward or outward movements of Na^+	

GFR, glomerular filtration rate; SW, seawater; FW, freshwater

SUMMARY

An RBCC (RING finger, B-box, and coiled-coil) protein was identified that belongs to the superfamily of zinc-binding proteins and is specifically expressed in the gill of eel, *Anguilla japonica*. Euryhaline fishes such as eels can migrate between freshwater and seawater, which is considered to be accomplished by efficient remodeling of the architecture and function of the gill, a major osmoregulatory organ. To identify molecules involved in such adaptive changes, I performed differential display using mRNA preparations from freshwater and seawater eel gills and obtained an RBCC clone among several differentially expressed clones. The clone encoded a protein of 514 amino acid residues with structural features characteristic of the RBCC protein; I therefore named it eRBCC (e for eel). eRBCC mRNA was specifically expressed in the gills with a greater extent in the gills of freshwater eels. Immunohistochemistry and *in situ* hybridization revealed that the expression of eRBCC is confined in particular epithelial cells of the gills including freshwater-specific lamellar chloride cells. The RING finger of eRBCC was found to have a ubiquitin ligase activity, suggesting an important regulatory role of eRBCC in the remodeling of branchial cells.

INTRODUCTION

RBCC proteins are a group of zinc-binding proteins that belong to the RING finger family. They are so called because they have an N-terminal RING finger motif defined by 7 cysteine and 1 histidine residues (C_3HC_4) followed by one or two additional zinc-binding domains (B-box), and a putative leucine coiled-coil region. The RING finger coordinates two zinc atoms and is found almost exclusively in the N-terminal position in RBCC proteins. The second motif or the B-box is defined by the consensus sequence CHC_3H_2 and binds one zinc atom. Members of the RBCC protein family include PML [7], TIF1 [8], KAP-1 [9], the MID1 gene product [10], XNF7 [11], RFP [12], SS-A/Ro [13], Rpt-1 [14], Staf50 [15], and HT2A [16] which are known to play important roles in regulating gene expression and cell proliferation [17-20]. Consistent with these functions, many of RBCC proteins have been defined as potential proto-oncogenes. I was interested in the RBCC protein family when I found a member of the family among cDNA clones that are differentially expressed between freshwater and seawater eels while attempting clarification of the mechanism of adaptation of euryhaline fishes. Euryhaline fishes can survive in both freshwater and seawater. Moving from freshwater to seawater or *vice versa* is expected to be accompanied by massive reorganization of the molecular architecture of gill cells or changes of their types. To understand the molecular basis for such extraordinary ability of adaptation, identification and characterization of regulatory proteins, such as RBCC family members, are essential.

The RBCC protein identified here is unique not only in its C-terminal sequence but also in its restricted expression: It is highly expressed in the gill but not in detectable amounts in other tissues and furthermore it is expressed much more highly in

freshwater than in seawater eels, suggesting that the eel RBCC may play an important role in the differentiation and maintenance of freshwater gill cells. In support of this potential regulatory role, I show here that the eel gill RBCC protein has an E3 ubiquitin ligase actitity. The ubiquitin system targets a wide array of short-lived regulatory proteins and incorporates into them a ubiquitin tag for degradation through a three-step mechanism involving ubiquitin activating (E1), conjugating (E2), and ligating (E3) enzymes [21].

EXPERIMENTAL PROCEDURES

Animal

Japanese eels (*Anguilla japonica*) weighing approximately 200 g were purchased from a local dealer. They were reared unfed in a freshwater tank for 2 weeks (freshwater-adapted eels). Some eels were transferred to a seawater tank and acclimated there for 2 weeks before use (seawater-adapted eels). The water temperature was maintained at 18--22°C. All eels were anaesthetized by immersion in 0.1% ethyl *m*-aminobenzoate methanesulfonate (MS222) before being killed by decapitation. The various tissues for RNA extraction were dissected out, snap-frozen in liquid nitrogen and stored at −80°C until use.

Differential display

Differential display was performed following the protocol of Liang and Pardee [22,23]. Total RNA was isolated by the guanidinium thiocyanate/cesium chloride method [24] from a pool of gill tissues from 5 freshwater- and 5 seawater-adapted eels, and then mRNA was prepared using an oligo(dT)-cellulose column (Amersham Pharmacia Biotech). One μg of mRNA was used for cDNA synthesis with a Superscript kit (Life Technologies, Inc.) together with a single arbitrary primer. Differential display PCR was performed using 5 ng of cDNA, 1 μM same arbitrary primer, 0.5 mM dNTPs, 0.7 MBq of [α-^{32}P]dCTP (Amersham Pharmacia Biotech), and 2.5 units of *Taq* polymerase (Takara). The mixture was cycled first at 94°C for 1 min, 36°C for 5 min, and 72°C for 5 min followed by 40 cycles at 94°C for 1 min, 60°C for 2 min, and 72°C for 2 min. An aliquot of each amplification mixture was subjected to electrophoresis in a 7.5%

polyacrylamide gel, exposed to an imaging plate for 8 h and the result was analyzed with a BAS-2000 image analyzer (Fuji Film). Differentially expressed bands of interest were extracted from the gel and reamplified and then cloned into pBluescript II vector (Stratagene). DNA sequence analysis from both strands was performed using a SequiTherm^{TM} cycle sequencing kit (Epicentre Technologies). The DNA sequence was compared with the GenBank^{TM}/EMBL/DDBJ databases using the BLAST network service at the National Center for Biotechnological Information.

Northern blot analysis

Poly(A)-rich RNA (3 μg) from a pool of gill tissues from 5 freshwater- and 5 seawater-adapted eels was denatured in a 2.2 M formaldehyde, 50% (v/v) formamide buffer and then separated on 1% agarose gel containing 2.2 M formaldehyde. Size-fractionated RNAs were then transferred to a nylon membrane (MagnaGraph, Micron Separations Inc.). The eRBCC cDNA was ^{32}P-labeled by random priming and hybridized to the RNA filters in 50% formamide, 5 × SSPE (SSPE, 0.15 mM NaCl, 1 mM EDTA, and 10 mM NaH_2PO_4, pH 7.4), 2 × Denhardt's solution, and 0.5% SDS for 16 h at 42°C. After hybridization, the membrane was rinsed twice in 2 × SSC (1 × SSC contains 0.15 mM NaCl and 0.015 M sodium citrate) containing 0.1% SDS for 30 min at 50°C, washed with 0.5 × SSC containing 0.1% SDS for 1 h at 55°C. The membrane was exposed to an imaging plate for 8 h and the result was analyzed with a BAS-2000 image analyzer.

Screening and sequencing

The freshwater-adapted eel gill cDNA library in λZAP II (Stratagene) was prepared as described [25]. The library was plated out at a density of 3×10^4 plaque-forming

units/150-mm plate. Phage plaques were lifted onto nitrocellulose filters (Schleicher & Schuell), and the filters were prehybridized for 2 h at 42°C in a solution containing 50% formamide, 5 × SSPE, 0.1% SDS, and 5 × Denhardt's solution. The probe was labeled with [α-^{32}P]dCTP using random primers. Hybridization was performed for 16 h at 42°C. To identify positive clones, filters were washed and then exposed to Kodak X-Omat film at -80°C overnight with intensifying screens. Positive plaques were isolated and rescreened after dilution. Conditions for secondary and tertiary screening were identical to primary screening. The obtained positive clones were excised with R408 helper phage (Stratagene) and sequenced using a SequiTherm^{TM} cycle sequencing kit.

Rapid amplification of cDNA ends (RACE) PCR

To obtain the 5' end of the eRBCC cDNA, 5'-RACE PCR was conducted using the 5'/3'-RACE kit (Roche Molecular Biochemicals). One μg of poly(A)-rich RNA from freshwater-adapted eel gill was reverse-transcribed using the gene-specific antisense primer, 5'-CTTGAAGTGCTCGGT-3', complementary to nucleotides 450--464 of the eRBCC cDNA sequence by AMV reverse transcriptase. First strand cDNA was purified and oligo(dA)-tailed according to the manufacturer's protocol. The resulting cDNA was then PCR-amplified using a second gene-specific antisense primer, 5'-ATCTCCTTCAGGGTGCGGTT-3', complementary to a eRBCC cDNA nucleotides 429--448 of the eRBCC cDNA and an oligo(dT) anchor primer supplied by the manufacturer. Second PCR was performed using a third gene-specific antisense primer, 5'-ATGTGCAGGCAGGGCCTCTT-3', complementary to nucleotides 408--427 of the eRBCC cDNA and a PCR anchor primer supplied by the manufacturer. The PCR products were cloned into pBluescript II vector. DNA sequence analysis was performed

using a SequiTherm^{TM} cycle sequencing kit.

RNase protection analysis

RNase protection assays were performed using an RPA II kit (Ambion) according to the manufacturer's protocol. A 540-bp PCR fragment of eRBCC cDNA (1233--1772) and a 138-bp PCR fragment of eel β-actin cDNA were subcloned into the pBluescript II vector and used to generate cRNA probes. The probes were synthesized with T7 RNA polymerase and an RNA transcription kit (Stratagene) in the presence of [^{32}P] UTP (Amersham Pharmacia Biotech). The RNA probe was treated with DNase I, purified by Sephadex G-50 chromatography and ethanol precipitation, and 1.7×10^2 kBq of the probe was hybridized to 10 μg of total RNA from pools of various tissues from 5 freshwater- or 5 seawater-adapted eels for 16 h at 42°C. After digestion with RNase A/T1, protected fragments were electrophoresed on 5% polyacrylamide, 8 M urea denaturing gels and exposed to an imaging plate for 16 h and the result was analyzed with a BAS-2000 image analyzer.

Transfer experiment

To examine the time-course changes in the levels of eRBCC mRNA following freshwater entry, seawater-adapted eels (n = 36) were transferred directly to freshwater and the gills were sampled from 6 eels on days 0, 1/8 (3 h), 1/2 (12 h), 1, 3, and 7 for RNase protection assay. Six seawater eels that were kept in seawater for 7 days served as time controls. The changes in the levels of Na^+,K^+-ATPase mRNA were also examined in parallel with those of RBCC. The data served as reference controls because its expression may be down-regulated in contrast to the expected up-regulation

of RBCC. The changes in plasma Na^+ concentration were monitored during the course of freshwater adaptation. The collected gill tissues were immediately frozen in liquid nitrogen, and total RNA was isolated as mentioned above. RNase protection assay performed with 10 μg of each RNA as described above. Optical densities of the protected fragments for each gill were measured and normalized to the β-actin bands. The mean normalized values were plotted ± S.E. The Student's *t*-test was used to determine the significance of any differences between two groups, $P < 0.05$ was considered significant.

Antibody production

A PCR fragment of the eRBCC cDNA (corresponding to amino acid residues 1--514) was subcloned into the bacterial expression vector pRSET-A (Invitrogen). After induction with 1 mM isopropyl-1-thio-β-D-galactopyranoside, the fusion protein was expressed in *Escherichia coli* strain BL21 and purified in a denaturing buffer (8 M urea, 50 mM Na_2HPO_4 and 300 mM NaCl, pH 7.6) by affinity column chromatography using Ni-NTA agarose (Qiagen) and dialyzed against phosphate-buffered saline (PBS: 100 mM NaCl, 10 mM NaH_2PO_4, pH 7.4) at 4°C. About 100 μg of the fusion protein emulsified in complete Freund's adjuvant (1:1) was injected into rats to raise polyclonal antibodies. The rats were injected 3 times at 2-week intervals and bled 7 days after third immunization.

Affinity purification of anti-eRBCC antibody

The polyclonal rat serum was purified on an affinity column. The affinity column was prepared by coupling 1 mg of His_6-cRBCC fusion protein to an Affi-Gel solid support,

according to the manufacturer's instruction (Bio-Rad) and then 10 mL of anti-eRBCC antiserum (diluted 1:10 in PBS) was applied to the column and incubate at 4°C for 24 h. The bound antibody was eluted with 10 mL of 100 mM glycine (pH 2.5) and dialyzed against PBS.

Cell culture and plasmid transfection

COS-7 cells were cultured in Dulbecco's modified Eagle's medium (Sigma) containing 10% fetal bovine serum and 100 units/ml penicillin. The cells were maintained in humidified atmosphere with 5% CO_2 at 37°C. The eRBCC cDNA was introduced into the pcDNA3 vector. Cells were transfected with the plasmid using LipofectAMINE (Life Technologies, Inc.) according to the manufacturer's instruction.

Western blotting

The COS-7 cells expressing eRBCC or mock transfected cells were washed three times with PBS and solubilized with Laemmli buffer. The cell lysate was separated by SDS-polyacrylamide gel electrophoresis (PAGE) and transferred onto polyvinylidene difluoride (PVDF) membrane. Nonspecific binding was blocked with 10% fetal bovine serum in TBS-T (TBS-T: 100 mM Tris/HCl, pH 7.5, 150 mM NaCl, and 0.1% Tween 20). The membrane was then incubated with the affinity purified anti-eRBCC antibody at 1:200 dilution overnight at 4°C. After washing the membranes in a TBS-T, blots were incubated with horseradish peroxidase-linked second antibody followed by enhanced chemiluminescence detection using the ECL-Plus reagent according to the manufacturer's instruction (Amersham Pharmacia Biotech).

Subcellular fractionation of eel gill

Subcellular fractionation of eel gill was performed by lysing gill cells peeled from removed eel gill with a Dounce homogenizer in buffer A (10 mM HEPES, 10 mM KCl, 1.5 mM $MgCl_2$, 0.5 mM dithiothreitol, pH 7.9), followed by cetrifugation at 1,000 × *g* for 10 min at 4°C. The pellet was saved as a crude nuclear fraction. The supernatant was further centrifuged at 10,000 × *g* for 30 min at 4°C to obtain mitochondrial fraction (pellet) and cytosol fraction (supernatant). Nuclei were purified essentially as described by Dignam *et al.* [26]. Briefly, the crude nuclear fraction was homogenized in buffer C (20 mM HEPES, 25% glycerol, 0.42 M NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM dithiothreitol, pH 7.9), and centrifuged at 10,000 × *g* for 30 min at 4°C (supernatant, nuclear fraction). Each fraction was separated by SDS-polyacrylamide gel electrophoresis (PAGE), and analyzed by western blotting described above for detection of eRBCC in the eel gill.

In situ hybridization

Freshwater-adapted eels were perfused with PBS containing 4% (w/v) paraformaldehyde. The gills were removed, immersed in the same fixative for 24 h at 4°C, and then soaked in PBS containing 20% (w/v) sucrose for 2 h at 4°C. The organs were embedded in Tissue Tek OCT Compound and frozen on a cryostat holder. Sections (10 μm) were prepared at −25°C in a cryostat and mounted on Vectabond-treated glass slides and dried in air for 1 h. The sections were postfixed in PBS containing 4% (w/v) paraformaldehyde, and immersed in 50% formamide, 5 × SSC at 55°C after washing with PBS. Digoxygenin (DIG)- or ^{33}P-labeled cRNA probe was synthesized with the same template and polymerase as those used for the RNase

protection analysis. Control sense probe was also synthesized in a similar way. The sections were hybridized with these probes (500 ng/ml) in 50% formamide, 5 × SSC at 55°C for 48 h. After hybridization, the sections were washed with 0.1 × SSC at 65°C for 1 h. The hybridized DIG-labeled cRNA was detected with anti-DIG alkaline phosphatase-conjugated antibody, and visualized as blue precipitates by the subsequent alkaline phosphate-catalyzed color reaction with 5-bromo-4-chloro-3-indoyl phosphate and nitroblue tetrazolium. For the ^{33}P-labeled cRNA detection, sections were dipped into a nuclear track emulsion (Konica NR-M2) at 42°C and the emulsion was developed after exposing at 4°C in the dark for 9 days.

Immunohistochemistry

Ten eels were first acclimated in seawater for 2 weeks and 5 of them were then transferred to freshwater. On day 7 after transfer, gills were removed from freshwater and seawater eels and fixed for 2 h in PBS containing 4% (w/v) paraformaldehyde at 4°C. After incubation in PBS containing 20% (w/v) sucrose for 1 h at 4°C, the specimen was frozen in Tissue Tek OCT Compound on a cryostat holder. Sections (5 μm) were prepared at –20°C in a cryostat and mounted on Vectabond-treated glass slides and dried in air for 1 h. After washing with PBS, sections were permeabilized by incubating in PBS containing 0.1% Triton X-100 at room temperature for 5 min and then incubation with PBS containing 0.3% (v/v) H_2O_2 for 30 min at room temperature. For staining, sections were incubated with affinity-purified anti-eRBCC antibody (1:200) or anti-eRBCC serum (1:2,000) or preimmune serum (1:2,000) or anti-eRBCC preabsorbed with the corresponding antigen (1:2,000) or anti-Na^+,K^+-ATPase α-subunit antiserum (1:10,000) [27] at 4°C overnight. Bound antibodies were detected by

incubation with biotinylated second antibody (diluted 1:200) and avidin-peroxidase conjugate using the Vectastain ABC kit (Vector Laboratories) following the manual supplied.

Immunofluorescence

Gills form freshwater-adapted eels (n = 5) were fixed for 4 h in PBS containing 4% (w/v) paraformaldehyde at 4°C, immersed in PBS containing 20% (w/v) sucrose for 1 h at 4°C, and frozen in Tissue Tek OCT Compound. Sections (7 μm) were cut and permeabilized as described above. After incubation for 1 h in PBS containing 2% (w/v) fetal bovine serum, sections were incubated with affinity-purified anti-eRBCC antibody (1:200) and anti-Na^+,K^+-ATPase α-subunit antiserum (1:10,000) [27] at 4°C overnight. Bound antibodies were detected by incubation with anti-rat IgG Cy3-conjugated (Jackson ImmunoResearch Laboratories; 1:400) and anti-rabbit IgG Alexa488-conjugated (Molecular Probes; 1:1,000) second antibodies together with Hoechst 33342 (Molecular Probes ; 100 ng/ml). Immunofluorescence microscopy was carried out using an Olympus IX70 microscope (Olympus).

In vitro ubiquitination assay

A Glutathione *S*-transferase (GST) fusion of eRBCC was expressed in *E. coli* and assessed for its ubiquitination activity *in vitro* as described [28,29] with some modifications. Reaction mixtures were assembled in 20 μl of a buffer containing 0.1 μg of rabbit E1, 1 μg of E2, 1 μg of GST-Ub, 25 mM Tris/HCl (pH 7.5), 120 mM NaCl, 2 mM ATP, 1 mM $MgCl_2$, 0.3 mM dithiothreitol, 1 mM creatine phosphokinase, 100 μM MG-132, and 100 ng of GST-eRBCC. E2s (UbcH2, UbcH5C, UbcH7, UbcH8, and

UbcH9) used in ubiquitination assay were expressed as recombinant proteins in *E. coli*. After incubation at 30°C for 4 h, the samples were processed for SDS-PAGE on 10% gels and Western blot with mouse monoclonal antibody to ubiquitin. As a negative control, ubiquitination assay with 2 mM *N,N,N',N'*-tetrakis(2-pyridylmethyl)-ethylenediamine (TPEN) was performed.

RESULTS

Identification of a novel RBCC protein by differential display

In a differential display using mRNA preparations from freshwater and seawater eel gills, I identified an RBCC protein as a potential regulator of differentiation of gill cells. A strong differentially displayed band of 1600 bp (data not shown) was subcloned into pBluescript II, amplified in *Escherichea coli*, and sequenced. Computer-assisted analysis of the sequence confirmed that the clone encodes a member of the family of RBCC proteins. The RBCC protein was named eRBCC (e for eel).

Cloning of full-length cDNA and its sequence analysis

After confirming its differential expression by Northern blot analysis (Fig. 3), a full-length eRBCC cDNA was isolated from an eel gill cDNA library that was constructed using mRNA from freshwater eel gills. Fig. 4 shows the nucleotide sequence of the longest clone and the deduced amino acid sequence. eRBCC consists of 514 amino acid residues and has motifs characteristic of the RBCC protein at the N terminus: a RING finger of the C_3HC_4 type; a B-box, another form of zinc finger; and a coiled-coil domain (Figs. 5 and 6). Although the third Cys of the consensus sequence of the B-box (CHC_3H_2) is not conserved in eRBCC (CHC_2H_2, Fig. 6), the zinc-coordinating Cys and His residues are conserved. The C-terminal domain exhibited significant similarity (62--63%) to the B30.2-like domains of other known members including newt PwA33 [30], frog Xnf7 [11], and mammalian RFP [12] (Fig. 5). The B30.2-like domain is a conserved region of ~170 amino acid residues usually found in the C-terminal position [5]. These structural features and the unique tissue distribution indicate that eRBCC is a

novel member of the C-terminal-domain-containing subgroup of the RBCC group of RING finger proteins.

Although the first Met codon is in a perfect Kozak consensus environment (GGCATGG) [31], no stop codon could be found in frame upstream of the start codon. Therefore I performed 5'-RACE to confirm the position of the initiator Met codon. Most of the RACE products terminated at the position almost identical to that of the longest cDNA clone, rendering the possibility of the existence of another ATG codon upstream of position +1 unlikely.

Confirmation of freshwater- and gill-specific expression by RNase protection analysis

Using total RNA preparations from various tissues of freshwater and seawater eels, I performed RNase protection analysis, a method capable of detecting specific RNA species with high sensitivity and accuracy [32,33], to determine the tissue distribution of eRBCC mRNA. Expression of the eRBCC message was highly restricted to the gill (Fig. 7). Compared to the levels in seawater eel gills, its levels in freshwater eel gills were much higher.

Time course of induction during freshwater adaptation

After transfer of seawater eels to freshwater, the expression of RBCC mRNA in the gill was induced and maximal induction occurred after 12 h to ~5 fold compared with the seawater level (Fig. 8A). Significant increases in RBCC mRNA continued thereafter for 7 days. The levels of RBCC mRNA did not change in eels kept in seawater for 7 days. In contrast to the up-regulation of RBCC mRNA, the levels of Na^+,K^+-ATPase

mRNA decreased gradually to a level that was about half the original seawater level (Fig. 8B). The high levels of Na^+,K^+-ATPase mRNA in seawater persisted for 7 days in time controls. Plasma Na^+ concentration decreased gradually and reached equilibrium within 7 days after transfer to freshwater, thereby confirming successful adaptation to freshwater environments (Fig. 8C).

Immunochemical characterization of eRBCC

To detect the eRBCC, I raised antiserum against recombinant eRBCC, purified it by affinity chromatography, and performed affinity purification of the antiserum. Western blot analysis was performed with three fractions (nucleus, cytosol, and mitochondria) in the three groups of eels, freshwater-adapted eels, seawater-adapted eels, and eels that were in freshwater for 12 h after being transferred from seawater. On this Western blot analysis, two specific bands (57 kDa and 180 kDa) were detected in the nuclear fraction. A 180-kDa band was seen in all nuclear preparations (Fig. 9, *lanes* 3, 6, and 9), but a 57-kDa band, the predicted molecular weight from eRBCC sequence, was only observed in the freshwater 12-h sample (Fig. 9, *lane* 6). These bands of 57 kDa and 180 kDa were recognized in COS-7 cells transfected with eRBCC construct (Fig. 9, *lane* 1), but not in mock transfected cells. The result suggests the eRBCC protein may be covalently modified with other protein(s) or become crosslinked among themselves.

In situ localization of eRBCC mRNA in eel gill

I carried out *in situ* hybridization in the freshwater-adapted eel gill with digoxygenin (DIG)- and ^{33}P-labeled cRNA probes. The positive signals were detected in the gill epithelial cells of freshwater-adapted eel with DIG- and ^{33}P-labeled probes (Fig. 10, A

and C). But in the gill of seawater-adapted eel, no positive signal was detected (data not shown). Control experiments with sense eRBCC cRNA probes resulted in negative staining (Fig. 10, B and D).

Immunohistochemical localization of eRBCC

Affinity purification of the antiserum was effective to eliminate non-specific staining of the cartilaginous support of the primary lamella, which was seen together with specific staining in the secondary lamella when the crude antiserum was applied to gill sections (Fig. 11A, *panels* a and b). The secondary lamella staining was absent when preimmune serum (Fig. 11A, *panel* c) or preabsorbed antiserum (Fig. 11A, *panel* d) was used. Using the purified antibody, I next performed immunohistochemistry on sections of freshwater and seawater eel gills to determine the type of cells expressing eRBCC. Serial sections were stained with anti-eRBCC and anti-Na^+,K^+-ATPase. In freshwater specimens, anti-eRBCC immunostaining was observed mainly in epithelial cells of the secondary lamella (Fig. 11, *panels* a and e). The staining pattern was reminiscent of that of freshwater-type chloride cells that have recently been shown to migrate from the basal area to the outer surface of the secondary lamella in salmon [34] and eel [4]. I therefore stained consecutive sections with an antiserum against Na^+,K^+-ATPase, a marker enzyme of chloride cells [1,2]. Significant overlapping was observed between the eRBCC-positive cells (Fig. 10B, *panel* e) and the chloride cells decorated with anti-Na^+,K^+-ATPase (Fig. 11B, *panel* g; arrowheads). In seawater eel gill sections, eRBCC signals were weak and less abundant (Fig. 11B, *panel* f).

Fig. 12 shows simultaneous immunofluorescence staining of freshwater eel gill sections with anti-eRBCC (Fig. 12B), anti-Na^+,K^+-ATPase (Fig. 12C), and the

DNA-selective dye Hoechst 33342 (Fig. 12D). As seen from the merged image (Fig. 12A), the majority of eRBCC appears to be present in the nucleus of the epithelial cells of the secondary lamella including the chloride cells and pavement cells whose nucleuses are labeled by arrows and double arrowheads, respectively, in Fig. 12D. The nucleus of the pillar cells was not stained with anti-eRBCC (arrowheads). The mechanism of nuclear localization of eRBCC remains to be clarified since it has no apparent nuclear localization signal.

Ubiquitin ligase activity of eRBCC

Since it has recently been realized that the RING finger motif has a general role in ubiquitination, I determined whether eRBCC has a ubiquitin ligase activity using recombinant proteins generated in *E. coli* that do not express components of the ubiquitin-conjugating system. When GST-eRBCC was mixed with UbcH5C, an E2 enzyme, and GST-Ub in the presence of rabbit E1, ubiquitinated products of higher molecular weights were detected (Fig. 13A, *lane* 2). The bands were not observed in control experiments with TPEN, a zinc-cheleting agent, suggesting that the ubiquitination reaction was mediated by the E3 action of eRBCC (Fig. 13A, *lane* 3). To determine the specificity of eRBCC, I next prepared a number of recombinant E2 enzymes and examined their interaction with eRBCC. Ubiquitination reaction was observed only in the case of UbcH5C, demonstrating that eRBCC is relatively specific to UbcH5C (Fig. 13B).

DISCUSSION

In the present study, I identified an eel mRNA species that encodes an RBCC protein (eRBCC), is specifically expressed in the gill, and is therefore considered to be involved in the differentiation and maintenance of gill cells. The gill cell-restricted and fresh water-enhanced expression of eRBCC, first suggested by differential display, was confirmed by Northern blot analysis (Fig. 3) and RNase protection analysis (Fig. 7). Immunohistochemistry and *in situ* hybridization study suggested that the eRBCC-expressing cells are mainly located in the outer surface of the secondary lamella (Fig. 10, 11). Colocalization studies with an antiserum against Na^+,K^+-ATPase, a marker protein for the chloride cells, further revealed a significant overlap between eRBCC-positive cells and Na^+,K^+-ATPase-positive cells. This is interesting in relation to the recent finding of Uchida *et al.* [34] and Sasai *et al.* [4]. They demonstrated that the chloride cells can be classified into two types based on the locations in the gill: filament chloride cells and lamellar chloride cells. The lamellar chloride cells are considered to play a pivotal role in freshwater adaptation since they appear in freshwater and disappear in seawater [4,34]. The chloride cells are mainly located in the gill and involved in osmoregulation of teleost fish. Reflecting their extraordinary power of ion transport, chloride cells are rich in mitochondria and Na^+,K^+-ATPase and their surface areas are tremendously increased by extensive invaginations of the basolateral membrane [1,2]. Although circumstantial, my results suggest that eRBCC plays a key role in the differentiation and maintenace of certain epithelial cells, at least some populations of the lamellar chloride cells, of the freshwater eel gills. Identification, by future studies, of the molecules with which eRBCC interacts is essential for

understanding the function of eRBCC.

eRBCC belongs to a newly emerging family of modular proteins consisting of a C_3HC_4-type RING finger motif, one or two B-box(es), and one or two coiled-coil region(s). Members of the RBCC family [35-37] can be classified into several groups based on the numbers and locations of the B-box and coiled-coil regions and also by the presence or absence of a C-terminal domain. The known members of the C-terminal domain-containing group to which eRBCC belongs include newt A33 [30], frog Xnf7 [11], and mammalian RFP [12] (Fig. 5). The fact that 1) all these proteins have been implicated in the regulation of cell differentiation and 2) among the members, the C-terminal regions are relatively highly conserved suggests that eRBCC also has a similar functional role.

The RING finger motif has recently been shown in many cases to function as an E3 ubiquitin ligase [38-41]. However, the RING finger of this subfamily of the RBCC family has not been characterized except a recent report on Efp, a target gene product of estrogen receptor α essential for estrogen-dependent cell proliferation and organ development [6]. In the present study, I demonstrated that eRBCC has an E3 activity, which is dependent on, among the E2s examined, UbcH5C, an E2 enzyme that is considered to be involved in the stress response and play a central role in the targeting of short-lived regulatory proteins for degradation [42]. The finding may open a new avenue leading to better understanding of the mode of action of not only eRBCC but also other members of the RBCC family through identification of their cellular substrates.

Concerning physiological roles of RING finger proteins in fishes facing osmotic stress, a paper has recently been appeared reporting identification of Shop21, a salmon

homolog of the E3 ubiquitin ligase Rbx1, whose expression is highly induced in branchial lamella when salmon is exposed to seawater [43]. Shop21 identified by Jacques *et al.* [43] and eRBCC identified here may be one of the essential regulators for seawater and freshwater adaptation of euryhaline fishes. The proteins may contribute to remodeling of the gill architecture and its maintenance by targeting, for degradation via the proteasomal pathway, a group of regulatory and structural proteins that are not necessary for adaptation to new osmotic environments.

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FIGURES FOR THE MAIN TEXT

(Fig. 3—Fig.13)

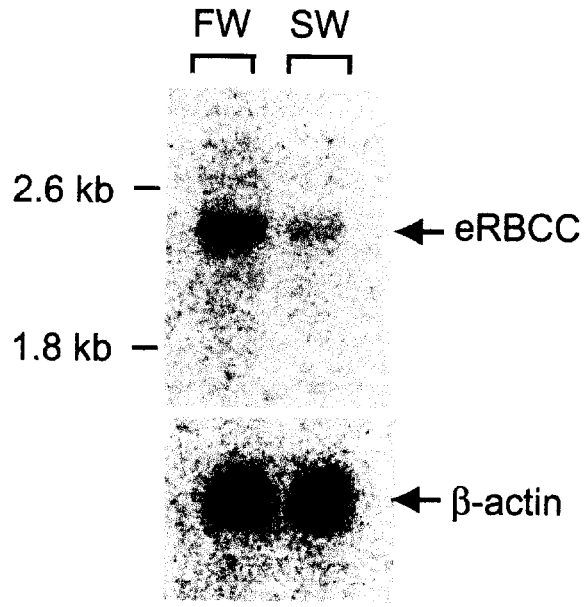

Fig. 3. Differential expression of eRBCC mRNA in gills of freshwater- and seawater-adapted eels. Northern blot analysis was performed using mRNA preparations from eels adapted to freshwater or seawater. Poly(A)-rich RNA (3 μg) from seawater and freshwater electrophoresed on a 1% agarose-formaldehyde gel, transferred to a nylon membrane, and hybridized with eRBCC ^{32}P-labeled cDNA probe. Position of 2.6 kb and 1.8 kb are as noted in figure. Hybridization to an eel b-actin probe demonstrated equal loading of the lanes. Data represent two separate experiments that yielded similar results.

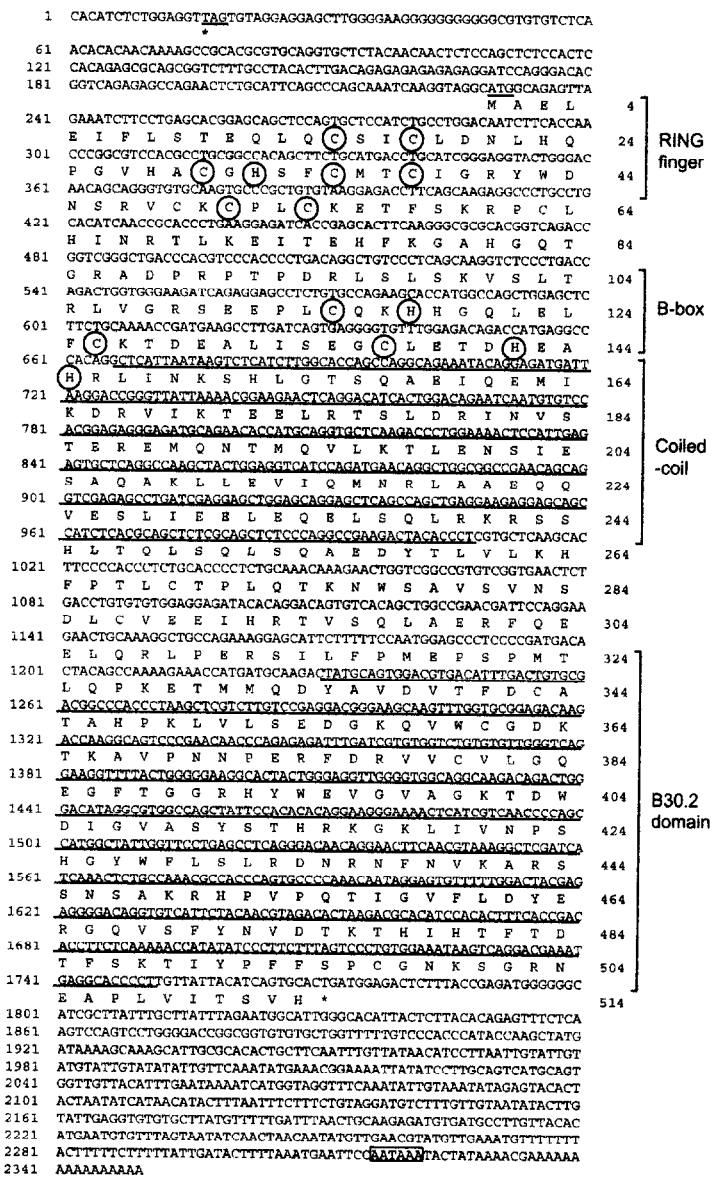

Fig. 4. Nucleotide and deduced amino acid sequences of eRBCC cDNA. The nucleotide sequence was derived from the longest clone. The first 98-bp nucleotides were isolated by 5'-RACE. The deduced amino acids are shown below their respective codons. Numbers to the right refer to the last amino acids on the lines, and the numbers to the left refer to the first nucleotides on the lines. The putative initiation codon 'ATG' and an upstream stop codon 'TGA' are underlined. Conserved cysteine/histidine residues in the RING finger domain and B-box domain are circled. The potential coiled-coil and B30.2 domain are underlined. Potential polyadenylation site in the 3'-untranslated region is boxed. Asterisks indicate stop codons.

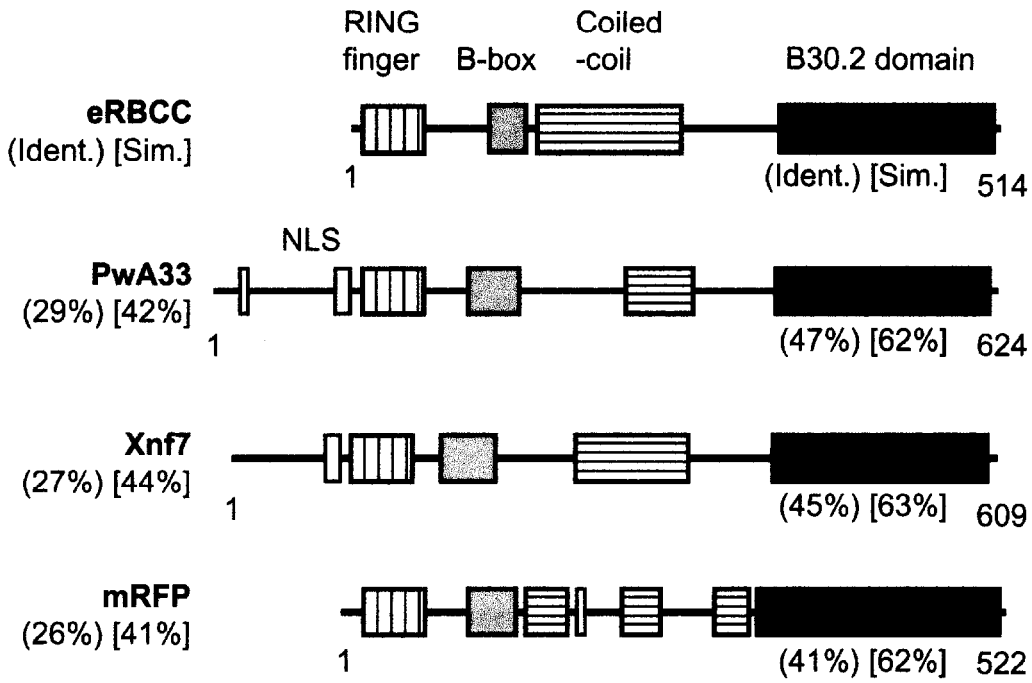

Fig. 5. Schematic representation of the relationship between eRBCC and several other RBCC proteins. The RING finger, B-box, coiled-coil, and B30.2 domains are shown as distinctive boxes. The overall identity (Ident.) and similarity [Sim.] of amino acids for each protein relative to eRBCC are shown under the name of the protein. The identity and similarity of the B30.2 domains are also shown. Proteins compared with eRBCC are PwA33 [30], Xnf7 [11], and mouse RFP (mRFP) [12]. NLS, nuclear localization signal (open box).

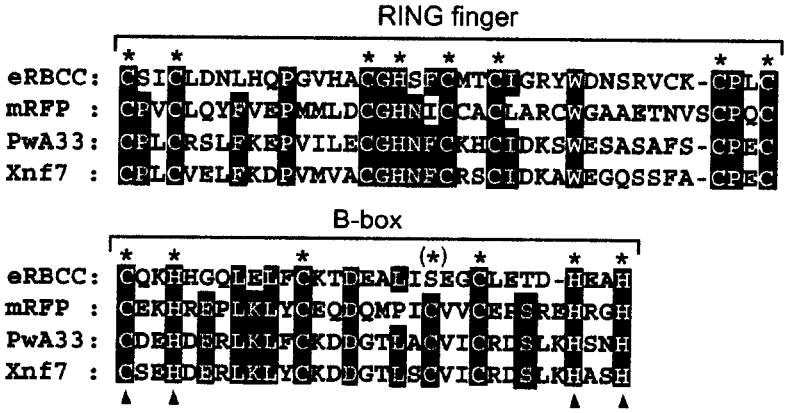

Fig. 6. Alignment of amino acid sequences of eRBCC, mRFP, PwA33, and Xnf7 proteins. The alignment of the amino acid sequence of the eRBCC RING finger domain and B-box domain with several members of the RBCC family is shown. The conserved Cys and His residues are shown with asterisks. The zinc-coordinating Cys and His residues of the B-box that binds one Zn atom are indicated by arrowheads.

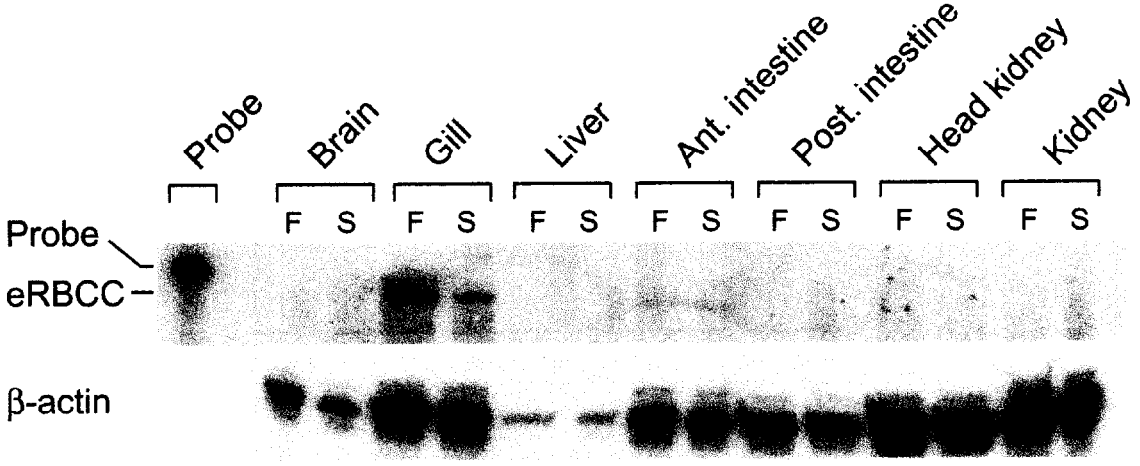

Fig. 7. eRBCC mRNA levels in various eel tissues in freshwater and seawater condition. Eels were adapted to freshwater or seawater for 2 weeks, and total RNA was isolated from the indicated tissues. An autoradiogram of an RNase protection assay (10 μg/lane) was performed with the indicated ^{32}P-labeled cRNA probe as described under "Experimental Procedures". In addition to the indicated tissues, I also analyzed total RNA preparations from the atrium, ventricle, stomach, and bladder, but they gave no signals (data not shown). Probe, labeled riboprobe alone; F, RNA preparation from freshwater-adapted eels; S, RNA preparation from seawater-adapted eels. A representative data set is shown from three separate experiments.

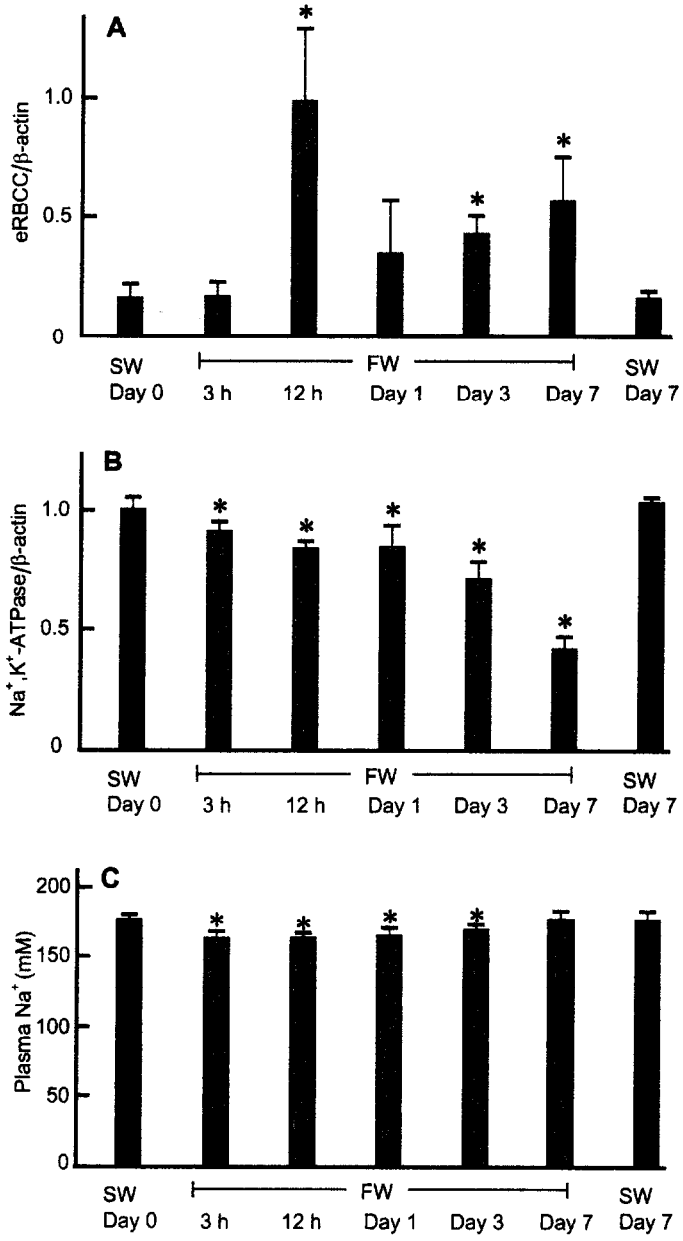

Fig. 8. Changes in the levels of eRBCC (A) and Na^+,K^+-ATPase (B) mRNA following transfer from seawater to freshwater. Seawater-adapted eels were transferred to freshwater and their RNA was isolated from gills of each eel separately (n = 4–6). RNase protection assay was performed as described under "Experimental Procedures". Optical densities of the protected fragments were measured and normalized to the β-actin bands. In C, plasma Na^+ concentrations are shown. The mean normalized values were plotted ± S.E. Asterisks indicate significant differences from the initial values (SW, day 0): $*P < 0.05$. SW, seawater; FW, freshwater.

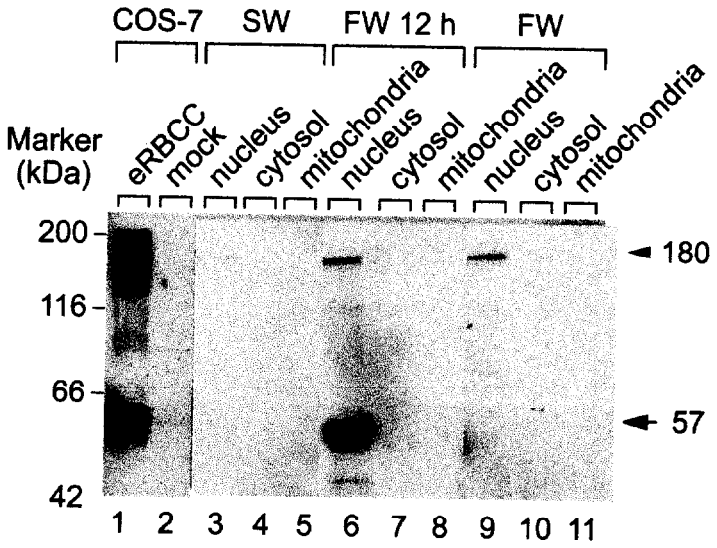

Fig. 9. Western blot analysis of eRBCC. Subcellular fractionation was performed with the gills from the freshwater- or seawater-adapted eels and the eels in freshwater for 12 h after seawater adaptation. COS-7 cells expressing eRBCC or mock transfected cells were solubilized with the Laemmli buffer. Each fraction of eel gill and the solubilized COS-7 proteins were analyzed by Western blotting as described under "Experimental Procedures". Two specific bands (57 kDa, indicated with arrow; 180 kDa, indicated with arrowhead) were observed in nuclear fraction of eel gill (*lanes* 3, 6, and 9). A 180-kDa band was seen in all nuclear preparations, but a 57-kDa band was only observed in the group in freshwater for 12 h (*lane* 6). These bands were recognized in COS-7 cells transfected with eRBCC construct (*lane* 1), but not in mock transfected cells. Positions of molecular weight markers are on the left.

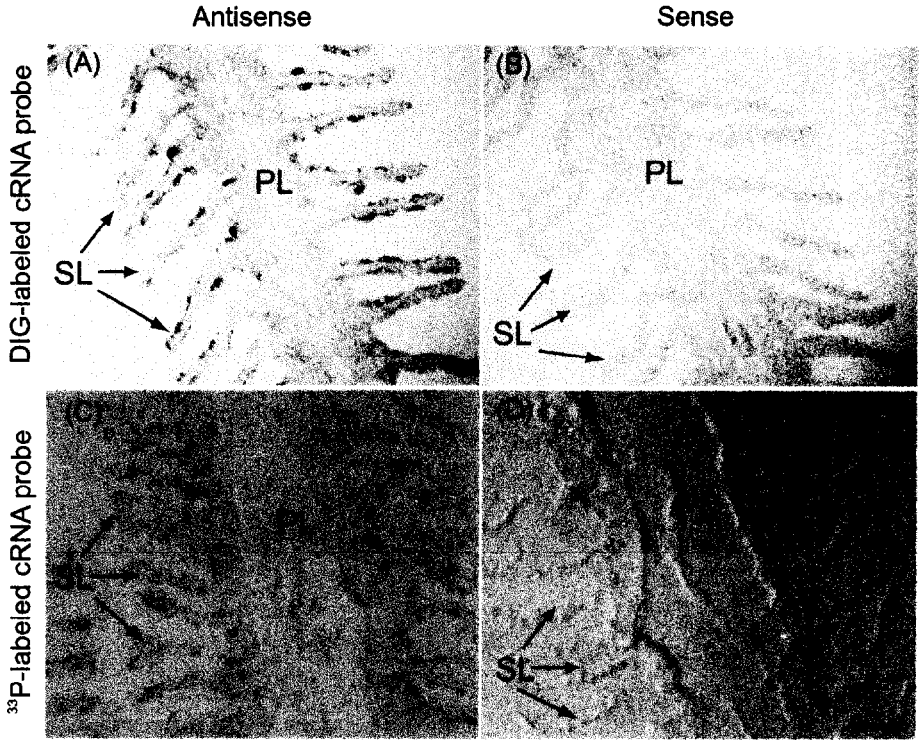

Fig. 10. Localization of eRBCC mRNA in the freshwater eel gill. *In situ* hybridization was performed in the freshwater eel gill. The sections were hybridized with digoxygenin-labeled antisense cRNA probe (A), control digoxygenin-labeled sense cRNA probe (B), ^{33}P-labeled antisense cRNA probe (C), and control ^{33}P-labeled sense cRNA probe (D) for eRBCC mRNA. PL, primary lamella; SL, secondary lamella. Scale bar represents 20 μm.

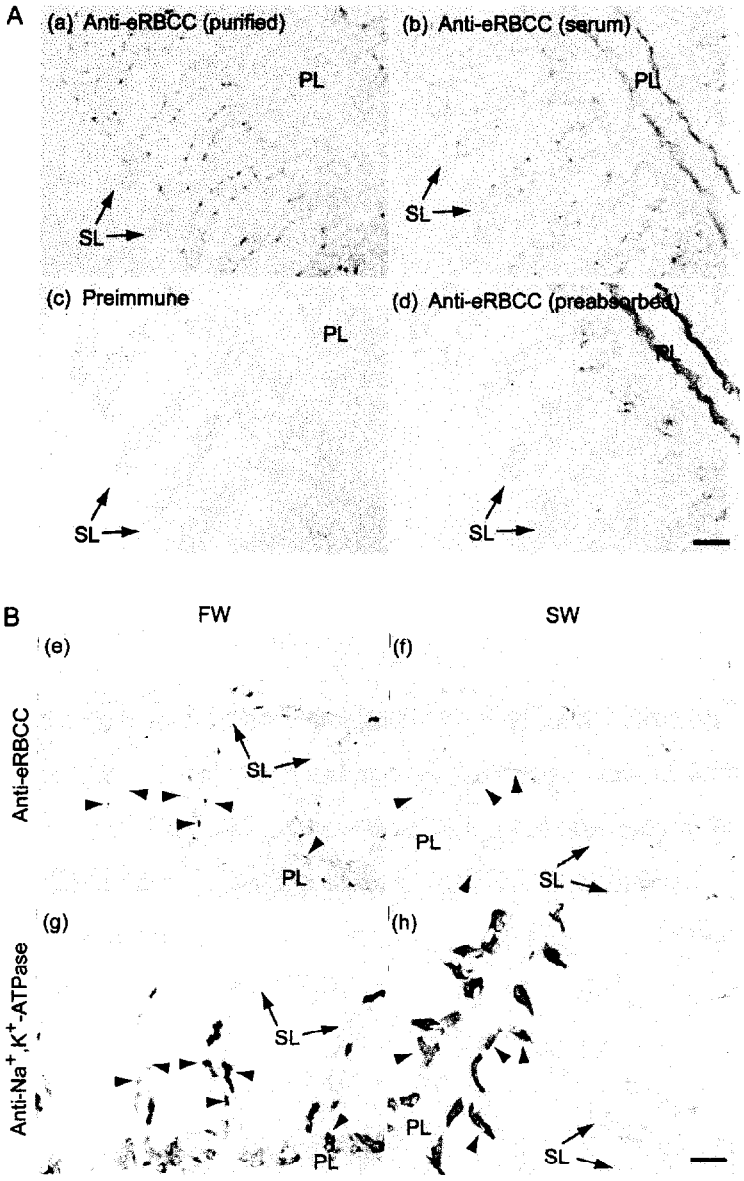

Fig. 11. Immunohistochemistry of eRBCC in freshwater and seawater eel gills. (A) Serial sections of freshwater eel gill were stained with affinity-purified anti-eRBCC antibody (a), antiserum against eRBCC (b), preimmune serum (c), and antiserum against eRBCC preabsorbed with the corresponding antigen (d). (B) Serial sections of freshwater (e, g) and seawater (f, h) eel gills were stained with affinity-purified anti-eRBCC antibody (e, f) and antiserum against Na^+,K^+-ATPase α-subunit (g, h). The arrowheads indicate the eRBCC positive chloride cells. PL, primary lamella; SL, secondary lamella. Scale bar represents 20 μm. Staining was repeated 10 times, with similar results, on gill sections from 5 different sets of freshwater and seawater eels.

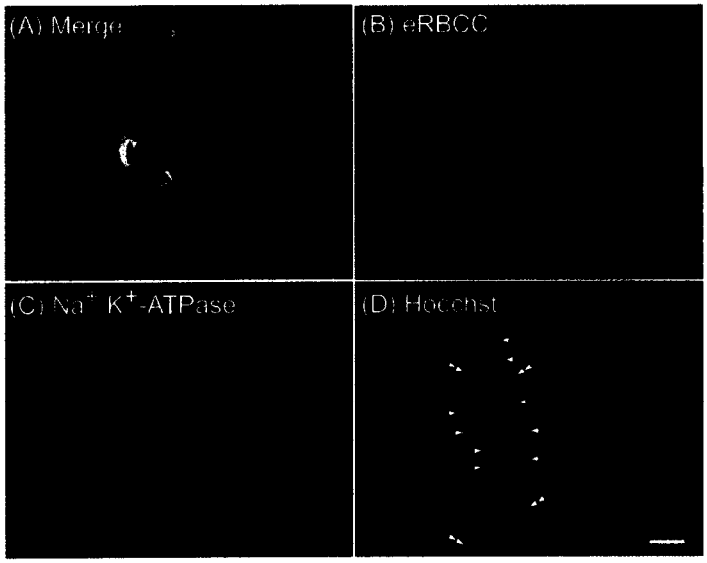

Fig. 12. Immunofluorescence localization of eRBCC and Na^+,K^+-ATPase in freshwater eel gill. Freshwater eel gill sections were stained with Cy3-conjugated antibody to eRBCC (B), Alexa488-conjugated antibody to Na^+,K^+-ATPase (C), and Hoechst 33342 (D). A merge of B, C, and D is shown in A. Scale bar represents 50 μm. Data represent three separate experiments. Similar results were obtained in two others.

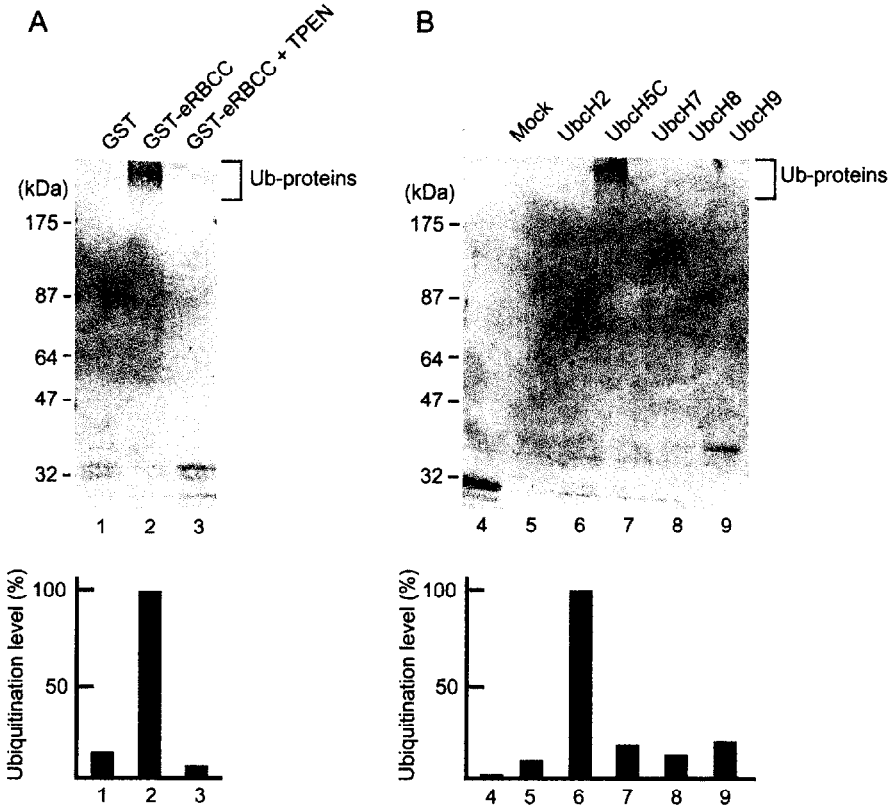

Fig. 13. E3 activity of eRBCC. (A) Demonstration of ubiquitin ligase (E3) activity of eRBCC. GST-eRBCC fusion protein was evaluated for its E3 activity in the presence of recombinant E2, UbcH5C, and GST-Ub with or without TPEN, a Zn^{2+}-chelating agent (*lanes* 1--3). (B) E2 preference of eRBCC proteins. Ubiquitination assay was performed with GST-eRBCC protein in the presence of the indicated E2 proteins (*lanes* 4--9). Bar graphs in A and B represent the results of quantitative analysis. The densities of high molecular weight bands (> 200 kDa) in *lane* 2 and *lane* 6, which reflect the amounts of ubiquitinated proteins, were taken as 100%.