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The cloning of eel osmotic stress transcription factor and the regulation of its expression in primary gill cell culture

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SUMMARY

In the present study, we aimed to clone an osmotic stress transcriptional factor (Ostf) from gill cells of Japanese eels. In addition, we measured its expression in Percoll^{TM}-gradient-isolated gill chloride (CC) and pavement (PVC) cells and determined the regulation of its expression in primary gill cell culture. Using degenerative primers and RACE techniques, we cloned a cDNA of 615 bp, encompassing the coding sequence of Ostf (204 amino acids). The cloned Ostf1 DNA sequence shared 84% DNA homology with the Ostf1 of tilapia. In general, the basal Ostf expression level was found to be significantly higher in CCs than in PVCs. In the direct transfer of fish from freshwater to seawater, a significant but transient induction of Ostf mRNA in CCs and PVCs was measured after 6 h of acclimation. Compared with gill CCs, the level of induction measured at PVCs was lower. In the seawater-to-freshwater transfer, no significant change in Ostf transcript levels was detected in either CCs or PVCs. To decipher the regulatory mechanism of Ostf expression, we conducted experiments using primary gill cell culture to specifically address the involvement of two putative osmosensors (i.e. intracellular ion strength/macromolecular crowding and cytoskeleton) in the regulation of Ostf expression. Hypertonic treatment using impermeable solutes (i.e. NaCl, 500 mOsmol l^{-1}) induced Ostf mRNA expression in 6 h, but no noticeable effect was measured using permeable solute (i.e. urea, 500 mOsmol l^{-1}). The induction was transcriptionally regulated and was abolished by the addition of organic osmolytes (i.e. betaine, inositol or taurine) into the culture media. Addition of colchicine (an inhibitor of microtubule polymerization) to hypertonic (with added NaCl, 500 mOsmol l^{-1}) cells reduced Ostf mRNA expression, suggesting that an increase in intracellular ionic strength and the integrity of the cytoskeleton are involved in the activation of Ostf mRNA expression in the cells. Collectively, the results of this study reveal, for the first time, the differential expression of Ostf in isolated CCs and PVCs. The resulting knowledge can shed light on how Ostf participates in hyperosmotic adaptation in fish gills.

Key words: chloride cell, organic osmolyte, osmosensor, pavement cell, Ostf, transcription factor.

INTRODUCTION

The capability of animal cells to maintain a constant cell volume is an essential prerequisite for cellular life (Chamberlin and Strange, 1989; Lang et al., 1998a). It is crucial for many cellular functions, including ion transport, cell migration, cell growth and cell death (Haussinger, 1996; Haussinger, 1998; Lang et al., 1998b). This regulatory process is particularly important in gill epithelia of euryhaline fish, as their gill cells are in direct contact with waters of different salinity. Although the physiological functions of fish gills have been studied extensively (Evans et al., 2005), very little is known about how gill cells sense and tolerate a large osmotic fluctuation. Major gaps exist in our understanding of how gill cells detect volume perturbations and transmit signals to activate volume regulatory mechanisms. In 2005, Kultz's group cloned two putative transcriptional regulators in gills of tilapia: the osmotic stress transcription factor 1 (Ostf1) and the basal transcription factor IIB (TFIIB), which are classified as the 'early hyperosmotically up-regulated proteins' (Fiol and Kultz 2005; Fiol et al., 2006). The regulation of Ostf1 mRNA expression in tilapia gills and primary gill cell culture was demonstrated to be activated by hypertonic treatment (Fiol and Kultz 2005; Fiol et al., 2006). Recently, the partial cDNA sequence of Ostf was cloned in black porgy (Choi and An, 2008). However, unlike the tilapia model, the expression of Ostf1 mRNA in the gills of black porgy was found to be activated only under hypo-osmotic stress. Until now, Ostf has only been cloned in two species of fish. In addition, the mechanism of regulation of Ostf expression has not been fully elucidated. In order to study the underlying mechanism in the regulation of its expression in branchial ion transporting cells, it would be necessary to clone the gene from a truly euryhaline fish. Further study in this area warrants our understanding of the evolutionary role as well as the functions of Ostf or related factors in osmoregulation.

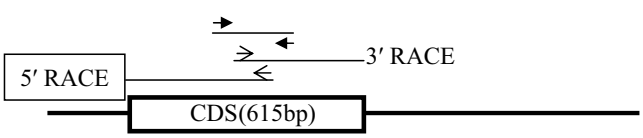
In the first part of the present study, we aimed to clone the Ostf cDNA from gills of Japanese eels. The expression profiles of Ostf mRNA upon freshwater-to-seawater transfer, and *vice versa*, were measured in Percoll^{TM}-gradient-isolated pavement cells (PVCs) and chloride cells (CCs). Additionally, we investigated the regulation of Ostf expression using primary gill cell culture. Our data indicate that the basal expression level of Ostf is significantly higher in gill CCs than PVCs. Hyperosmotic acclimation significantly activated Ostf expression in CCs. Hypo-osmotic adaptation, however, had no obvious effect on Ostf mRNA levels. Using primary cell culture, we demonstrated that Ostf gene activation is influenced by an increase in intracellular ionic strength and affected by the integrity of the cytoskeleton.

MATERIALS AND METHODS

Animals and gill cell isolation

Japanese eels (*Anguilla japonica* Temminck and Schlegel) weighing between 500 and 600 g were reared in fiberglass tanks supplied with

A



Degenerative Primers	Sources
5-AGTGCCTCYGGWGCYAGYGT-3 (forward) 5-TGGAACTTYTCCAGYTGYTC-3 (reverse)	Tilapia Ostf1 (AY679524) human and mouse TSC22 domain family sequences (NM 004089; NM 010286)
Gene Specific Primer (GSP)	
5-TCCGCCAGCTCCTTGATTTG-3	For 5 RACE
5-AGCAGGCAATGGATCTTGTGAA-3	For 3 RACE

B

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1-   M   S   D   E   E   C   R   S   P   I   G   L   D   C   C   S   C   C   L   D
   ATG TCG GAC GAG GAG TGC CGG TCT CCC ATT GGC CTG GAC TGC TGC AGC TGC TGC CTG GAC  -60
21-  L   A   N   G   C   D   A   V   P   G   R   P   G   L   N   A   M   G   S   P
   CTG GCA AAC GGC TGC GAC GCA GTG CCC GGC CGC CCG GGC CTC AAC GCC ATG GGC AGC CCC  -120
41-  T   V   T   H   F   R   Q   L   R   D   Q   L   M   F   Q   N   L   N   T   D
   ACC GTC ACC CAC TTT CGG CAG CTC CGG GAC CAG CTG ATG TTC CAG AAC CTC AAC ACG GAC  -180
61-  K   L   N   N   I   M   R   Q   D   S   L   E   S   V   V   R   D   P   C   F
   AAG CTG AAC AAC ATC ATG CGG CAG GAC TCC CTG GAG TCG GTG GTG CGG GAC CCC TGC TTC  -240
81-  L   L   N   E   G   I   C   N   S   N   I   D   Q   T   M   L   S   I   L   L
   CTA CTC AAC GAG GGC ATC TGC AAC AGC AAC ATC GAC CAG ACC ATG CTG TCC ATT CTG CTC  -300
101- Y   F   H   S   A   S   G   A   S   V   V   A   I   D   N   K   I   E   Q   A
   TAC TTC CAC AGT GCC TCT GGT GCT AGC GTT GTG GCG ATT GAC AAC AAG ATC GAG CAG GCA  -360
121- M   D   L   V   K   N   H   L   M   Y   A   V   R   E   E   V   E   I   L   K
   ATG GAT CTT GTG AAG AAC CAC CTG ATG TAC GCA GTC CGG GAG GAG GTG GAG ATT CTG AAA  -420
141- E   Q   I   K   E   L   A   E   K   N   N   Q   L   E   R   E   N   S   L   L
   GAG CAA ATC AAG GAG CTG GCG GAG AAG AAC AAC CAG CTG GAG CGG GAG AAC AGC CTG CTG  -480
161- K   N   L   A   S   P   E   Q   L   E   K   F   Q   S   R   L   P   P   E   E
   AAG AAC CTG GCC AGC CCA GAG CAG CTG GAG AAG TTC CAG TCC CGT CTG CCC CCG GAG GAG  -540
181- A   Q   L   A   Q   S   Q   P   V   D   S   P   D   Q   D   V   L   R   S   A
   GCC CAG CTC GCG CAG AGC CAG CCG GTG GAC TCG CCT GAC CAG GAC GTC CTG CGC AGC GCC  -600
201- G   S   A   V   *
   GGC TCC GCC GTG TGA
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C

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Tilapia          1 MSDDDCLSPI GLDCCSCCLD LANGCDESIS SSPAQGLGTT GGLPASPTSP 50
Japanese eel     1 MSDEECRSPI GLDCCSCCLD LANGCD-AVP GRP--GLNAM G-------SP 50

Tilapia         51 TVNHFRQLRN QLMYQNLNTD KLNNIMRQDS LESVVRDPCF LLNEGICNSN 100
Japanese eel    51 TVTHFRQLRD QLMFQNLNTD KLNNIMRQDS LESVVRDPCF LLNEGICNSN 100

Tilapia        101 IDQTMLSILL FFHSASGASV VAIDNKIEQA MDLVKNHLMY AVREEVEILK 150
Japanese eel   101 IDQTMLSILL YFHSASGASV VAIDNKIEQA MDLVKNHLMY AVREEVEILK 150

Tilapia        151 EQIKELAEKN NQLERENYLL KNLASPEQLE KFQSRIPTDV LLPLDNQNIQ 200
Japanese eel   151 EQIKELAEKN NQLERENSLL KNLASPEQLE KFQSRLPPE- ----EAQLAQ 200

Tilapia        201 GTPEHQQQQQ QQTCNHSTGS AV*
Japanese eel   201 SQPVDSPDQD VL---RSAGS AV*
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Fig. 1. The cloning of eel Ostf cDNA. (A) Strategy for cloning the eel Ostf sequence. (B) DNA sequence of the 615 bp eel Ostf cDNA and the deduced amino acid sequence. (C) Comparison of amino acid sequences of the cloned eel Ostf and tilapia Ostf1 (GenBank accession no. AY679524). The mismatch or deleted residues are underlined. The conserved TSC-22 domain is bold.

charcoal-filtering aerated tapwater or seawater at 18–20°C under a 12 h:12 h L:D photoperiod for at least 3 weeks. The freshwater-adapted fish (N=12) were then transferred directly to seawater tanks, while seawater-adapted fish (N=12) were transferred directly to freshwater. Freshwater-to-freshwater (N=12) and seawater-to-seawater transfer control experiments (N=12) were conducted as well. The transferred fish [at days 0.25 (6 h), 1, 3 and 7] were sampled (three fish for each time point). The fish were anesthetized and perfused with phosphate-buffered saline (PBS) (pH 7.7) to remove blood cells from gills. Gill arches were excised and washed, and then were cut into small fragments and subjected to two cycles of tryptic digestion (0.5% trypsin + 5.3 mmol l^{-1} EDTA), each for 20 min at room temperature in a rotator (300 r.p.m.). The cell suspension was then filtered, washed and finally resuspended in 1.6 g ml^{-1} Percoll™ solution for PVC and CC isolation (Wong and Chan, 1999). The identity of the isolated CCs was confirmed by mitochondria staining (Tse et al., 2006). For real-time PCR analysis, the isolated gill cells were dissolved in TRIzol® Reagent (Invitrogen, Carlsbad, CA, USA) for total RNA extraction.

Cloning of eel Ostf cDNA

Purified gill RNA with an A_{260}/A_{280} ratio of 1.8–2.0 was used. Briefly, 0.5 μg of total cellular RNA was reverse transcribed (iScript; Bio-Rad, Hercules, CA, USA). Degenerative primers for Ostf were designed on the basis of the tilapia Ostf1 (AY679524), human (NM 004089) and mouse (NM 010286) TSC22 domains (Fig. 1A). The 3′ and 5′ ends of the target sequences were amplified using gene-specific primers (GSPs) according to the DNA sequences obtained from the PCR product amplified by the degenerative primers and the 3′ and 5′ RACE kits (Gibco/BRL, Gaithersburg, MD, USA; Invitrogen). PCR product was resolved by electrophoresis in a 2% agarose gel and was gel purified (QIAEX II agarose gel extraction kit; Qiagen, Valencia, CA, USA), then cloned into pCRII TOPO® (Invitrogen) for DNA sequencing by an outside vendor (TechDragon, Hong Kong).

Primary gill cell culture

The culture was established using gill filaments obtained from freshwater-adapted eels. Single-seeded preparations were used, as described in our previous study (Tse et al., 2007). After tryptic digestion of the gill cells, the cell suspension was then filtered and washed. The cells were resuspended in Leibovitz's L-15 medium (Gibco; Invitrogen) supplemented with 5% fetal bovine serum (FBS; HyClone®; Perbio Sciences, Logan, UT, USA), 1% penicillin/streptomycin, 1% gentamycin (Gibco; Invitrogen) and seeded at a density of 2×10^6 cells cm^{-2} onto collagen-coated culture plates (Iwaki, Chiba, Japan). The cells were incubated at 22°C in a growth chamber with humidified air. One day after seeding, each well was rinsed with PBS to remove mucous and unattached cells. The cells were then exposed to hypertonic stress and/or drug treatment. Hypertonic stress was induced by either the addition of (1) membrane-nonpermeable solute (NaCl; Sigma, St Louis, MO, USA) or (2) membrane-permeable solute (urea; Sigma) to the medium, making its osmolarity 500 mOsmol l^{-1}. In addition, in some experiments the cells were cotreated with actinomycin D (Act D, 1 μmol l^{-1}) (Calbiochem, Darmstadt, Germany) to determine if the effect was transcriptionally dependent. To test if the gene induction by hypertonicity can be modulated by organic osmolytes, the cultured cells were exposed to media containing high NaCl (final osmolarity of 500 mOsmol l^{-1}) and, at the same time, with or without 5 mmol l^{-1} inositol, betaine or taurine (Sigma). To test the involvement of cytoskeleton in Ostf activation, the hypertonic-exposed cells were co-treated with colchicine (100 μmol l^{-1}; Calbiochem). After 6 h of incubation, total RNA was extracted for the measurement of Ostf and GAPDH mRNA levels.

Real-time PCR analysis

Purified sample RNA with an A_{260}/A_{280} ratio of 1.8–2.0 was used. Briefly, 0.5 μg of total cellular RNA was reversed transcribed (iScript; BioRad). PCR reactions were conducted with the iCycler

iQ real-time PCR detection system using iQ™ SYBR® Green Supermix (Bio-Rad). Primers for Ostf (TCCGCCAGCTCCTTG-ATTTG-forward, AGCAGGCAATGGATCTTGTGAA-reverse) (Tse et al., 2007) and GAPDH (GCGCCAGCCAGAACATCATC-forward, CGTTAAGCTCGGGGATGACC-reverse) (GenBank accession no. AB075021) were used. The PCR products were cloned into pCRII TOPO® (Invitrogen) and subjected to dideoxy sequencing for verification. The copy number of the transcripts for each sample was calculated in reference to the parallel amplifications of known concentrations of the respective cloned PCR fragments. Standard curves were constructed and the amplification efficiencies were approximately 0.9–0.95. The occurrence of primer-dimers and secondary products was inspected using melting curve analysis. Our data indicated that the amplification was specific. There was only one PCR product amplified for each individual set of primers. Control amplification was done either without reverse transcriptase or without RNA.

Statistical analysis

All data are presented as means ± s.e.m. Statistical significance is tested by Student's *t*-test. Groups were considered significantly different if $P<0.05$.

RESULTS

Cloning and characterization of eel Ostf in gills

The open reading frame of eel Ostf cDNA is 615 bp in length, coding for 204 amino acids with a TGA stop codon (Fig. 1B). Multiple sequence alignment with the most similar DNA sequences and proteins was performed using BLAST. The cloned sequence shared 84% DNA sequence homology with tilapia Ostf1 (GenBank accession no. AY679524). The eel's amino acid sequence is shorter than that of the tilapia (222 amino acids) and shows some deletions of amino acids at the N- and C-terminals of the protein (Fig. 1C). Nevertheless, the two amino acid sequences share about 90% homology and both contain the TGF-β-stimulated clone 22 (TSC-22) domain.

Differential expression of Ostf mRNA in gill PVCs and CCs after transfer

To investigate whether Ostf is differentially regulated in freshwater PVCs and CCs, we isolated the cells by Percoll™ gradient centrifugation and measured Ostf mRNA level using real-time PCR assay. In freshwater-adapted eels, gill CCs expressed a significantly higher (~4 times) level of the transcript than that of the PVCs (Fig. 2A). To examine the effect of hypotonic and hypertonic stress on gill Ostf expression levels, freshwater-to-seawater and seawater-to-freshwater transfer experiments were conducted. In the freshwater-to-seawater transfer experiment, Ostf transcript levels in CCs and PVCs increased significantly by 4- and 0.8-folds, respectively, at 0.25 days post-transfer, after which they decreased back to basal levels (Fig. 2B). After transfer of the fish from seawater to freshwater, there was no obvious change in Ostf expression levels in either CCs or PVCs (data not shown). No significant change in Ostf transcript level was observed in the freshwater-to-freshwater and seawater-to-seawater transfer experiments (data not shown).

Regulation of Ostf mRNA expression in primary gill cell culture

To decipher the underlying mechanism in the regulation of Ostf expression, the primary gill cell model was adopted for the investigation. Hypertonic treatment of the cells using a

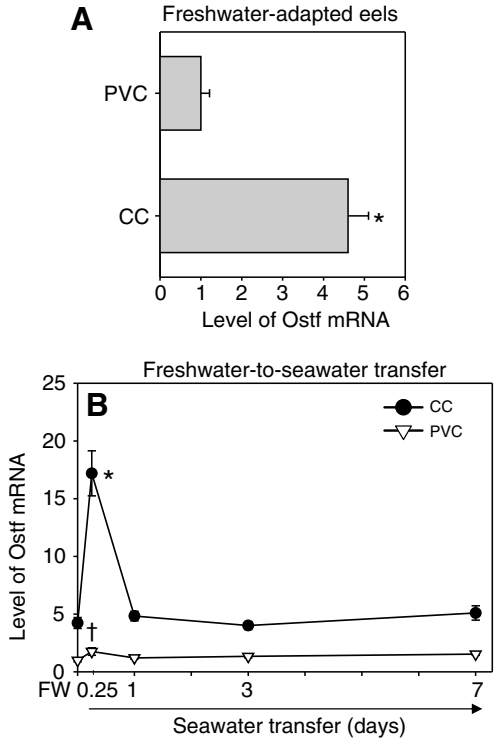

Fig. 2. The expression levels of Ostf mRNA in Percoll™-gradient-isolated gill pavement cells (PVCs) and chloride cells (CCs). Gill epithelia were dissected and digested; the cell suspension was centrifuged in Percoll™ gradient solution. The respective gill cell type was isolated, and total RNA was extracted and reverse transcribed for real-time PCR assay. (A) Comparison of Ostf expression levels in isolated freshwater PVCs and CCs. *$P<0.05$ compared with the PVC. (B) Change in Ostf mRNA expression levels in the gill PVCs and CCs isolated from fish at day 0.25, 1, 3 and 7 of seawater acclimation. A significant induction of Ostf mRNA in gill CCs after 0.25 days of seawater acclimation was measured. *$P<0.05$ compared with freshwater CCs. A small but significant increase of Ostf mRNA in isolated gill PVCs after 0.25 days of seawater acclimation was noted. †$P<0.05$ compared with the isolated freshwater PVCs. The results were obtained from four independent experiments.

nonpermeable solute (500 mOsmol l^{-1} NaCl) for 6 h significantly induced Ostf mRNA expression (Fig. 3A). Interestingly, hypertonic stress imposed by a permeable solute (urea) had no observable effect on the Ostf transcript level. The data suggest that the increase in intracellular ion strength is important in the induction of Ostf expression. The induction of Ostf expression was transcriptionally dependent, as revealed by co-treatment with Act D (Fig. 3B).

Attenuation of Ostf mRNA expression by organic osmolytes and colchicine

It is well known that cells respond to volume perturbations by modulating the transport of inorganic and organic osmolytes in the processes of regulatory volume change (Lang et al., 1998a; Strange, 2004; Wehner et al., 2003). The alteration in the composition of intracellular organic osmolytes is important to relieve volume perturbation. We tested this hypothesis by investigating whether an accumulation of organic osmolytes can attenuate the hypertonic stress-induced Ostf expression. In this regard, Ostf mRNA was measured in cells exposed to hypertonic medium (NaCl, 500 mOsmol l^{-1}) with or without the addition of 5 mmol l^{-1} of

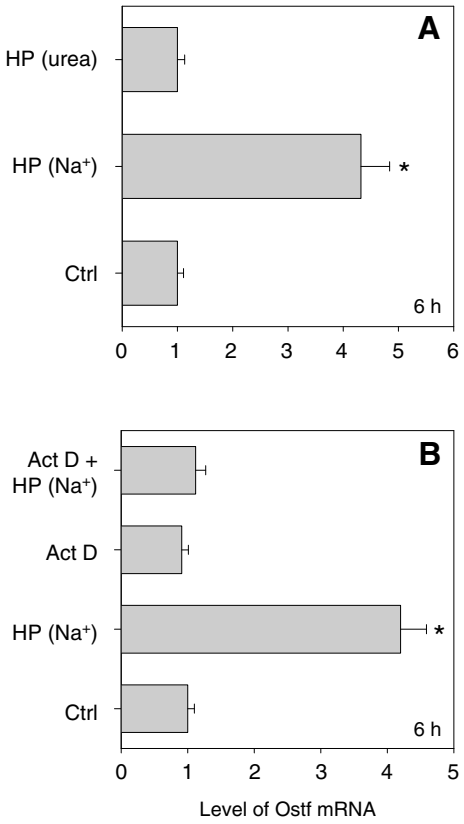

Fig. 3. Induction of Ostf mRNA expression levels in primary gill cell culture. Primary gill cells were grown overnight in isotonic medium (320 mOsmol l^{-1}) and were then subjected to hypertonic treatments and/or actinomycin D (Act D) treatment. After 6 h (0.25 days) of treatment, total RNA was extracted and reverse transcribed for real-time PCR assay.
(A) Hypertonicity was achieved by the addition of either (1) membrane-nonpermeable solute (NaCl) or (2) membrane-permeable solute (urea) to the medium, making its osmolarity 500 mOsmol l^{-1}. A significant induction of Ostf mRNA expression was observed in the hypertonic condition prepared by the addition of NaCl. (B) Effect of Act D on hypertonicity-induced Ostf mRNA expression. Hypertonicity and Act D cotreatment abolished the induced Ostf transcript levels. *P<0.05 compared with the control cells. The results were obtained from five independent experiments.

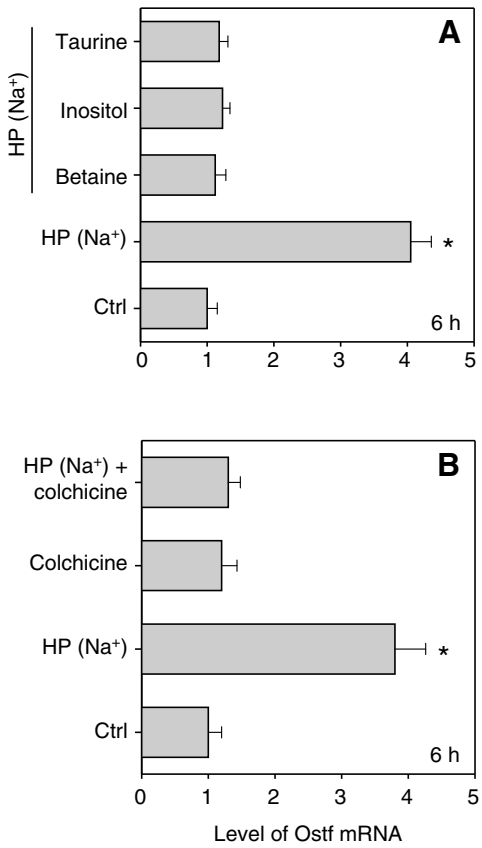

Fig. 4. Effects of organic osmolytes or colchicine on Ostf mRNA expression levels in primary gill cell culture. Primary gill cells were grown overnight in the isotonic medium (320 mOsmol l^{-1}) and were then subjected to hypertonic treatment (NaCl, 500 mOsmol l^{-1}) with the addition of (A) either 5 mmol l^{-1} of inositol, betaine or taurine or (B) 100 μmol l^{-1} colchicine. After 6 h of treatment, total RNA was extracted and was reverse transcribed for real-time PCR assay. Note that the cotreatment of the hypertonicity-exposed cells with the organic osmolytes or colchicine abolished the induced Ostf mRNA expression. *P<0.05 compared with the control cells. The results were obtained from five independent experiments.

inositol, betaine or taurine. Our data demonstrated that the hypertonicity-induced Ostf mRNA expression was attenuated by the addition of inositol, betaine or taurine in the culture medium (Fig. 4A).

In our previous study, we demonstrated that hypertonic treatment (NaCl, 500 mOsmol l^{-1}) of the gill cells caused cell shrinkage, probably by affecting membrane tension (Tse et al., 2007). Thus, it would be interesting to know if the induction of the Ostf transcript is related to cytoskeleton organization. The treatment of the hypertonic-treated cells (NaCl, 500 mOsmol l^{-1}) with colchicine (an inhibitor to microtubule polymerization) abolished the induction of Ostf expression (Fig. 4B).

DISCUSSION

Euryhaline fish have evolved highly adaptive osmoregulatory organs to deal with large fluctuations in water salinity. The capability of gill cells to undergo rapid cellular and molecular remodeling determines the rate and the direction of transepithelial ion transport in either the freshwater or seawater environment. Owing to the differing nature of osmotic stress in freshwater and seawater environments, osmosensing is a critical survival mechanism for gill cell remodeling (Fiol and Kultz, 2007). In the present study, we attempted to address this issue by cloning Ostf cDNA in gills of Japanese eels and determining its expression profile in the two major types of ion-transporting gill cells (i.e. CC and PVC) of acclimating fish. Furthermore, using the primary gill cell culture model, we deciphered the possible mechanisms in the regulation of Ostf expression.

The cloned eel Ostf shows high DNA and amino acid sequence homologies with those of tilapia (AY679524). The TSC-22 family signature sequence is identified in the eel Ostf cDNA. In the mammalian system, the TSC-22-domain-containing transcripts were reported to be important in protecting mouse kidney cells from osmotic stress (Fiol et al., 2007). PCR primers were designed according to the cloned eel Ostf sequence and were used for the analysis of its expression. In the first part of the study, we conducted *in vivo* experiments in which we compared the expression of Ostf mRNA in the Percoll^{TM}-gradient-isolated gill CCs and PVCs of the

fish acclimating in either the freshwater or seawater condition. The identification of these two cell types was on the basis of the evidence reported in our previous studies (Tse et al., 2006; Wong and Chan, 1999). Our data indicated that the basal levels of Ostf transcript were significantly higher in CCs than in PVCs. In the freshwater-to-seawater transfer experiments, the Ostf mRNA expression levels in CCs and PVCs increased significantly after 6 h and then decreased to the basal level from day 1 onwards. This observation indicated that the induction of Ostf is fast but transient. Comparatively, the induction was more substantial in gill CCs than in PVCs. In the course of seawater-to-freshwater transfer, there was no significant change in Ostf mRNA expression measured in either CCs or PVCs. This is comparable with the results reported in the tilapia model, which indicated only hypertonic stress-induced Ostf1 expression (Fiol and Kultz 2005; Fiol et al., 2006). It is believed that the modulation in the expression of the ion transporters and channels is induced by immediate early gene (IEG) transcription factors (Fiol and Kultz, 2005). When we compared the time windows of gene induction in gill cells of seawater-acclimating eels, our previous study demonstrated that most of the ion transporters increased their expression considerably 24 h after transfer to seawater (Tse et al., 2006). The early induction of Ostf expression (at 6 h of acclimation), which preceded the expression of ion transporters/channels (at 24 h of acclimation), suggests that Ostf is involved in the regulation of hyperosmotic responses in the gill cells (e.g. the expression of ion transporters or channels). The data demonstrate that Ostf was induced by hypertonic stress and its expression may play an important role in fish hyper-osmoregulation in the seawater environment.

It is known that a profound alteration in cell volume affects intracellular ionic strength (macromolecular crowding), cell membrane tension and, consequently, integrity of the cytoskeleton architecture (Lang et al., 1998a; Wehner et al., 2003). Although a considerable number of studies have been carried out to search for osmosensing mechanisms in the mammalian system, the conclusive model of the molecular osmosensors in animal cells has not yet been confirmed. Recently, our group has reported that there was an activation in the process of 'regulatory volume increase' as well as an increase in Ostf mRNA expression in gill cells after hypertonic (500 mOsmol l^{-1}) treatment (Tse et al., 2007). Hence, Ostf expression is thought to be one of the downstream targets in hyperosmotic responses. In the second part of the present study, we further determined that Ostf expression is induced in the hypertonic solution prepared using NaCl but not using urea. This observation indicates that Ostf activation is exerted by an increase in intracellular ionic strength and is directly coupled to one of the putative osmosensors, macromolecular crowding (Lang et al., 1998a; Wehner et al., 2003). To further elucidate this possibility, the hypertonic-treated cells were incubated in media containing organic osmolytes (i.e. betaine, taurine or inositol). This treatment presumably decreases the intracellular ionic strength by stimulating the cellular accumulation of organic osmolytes (Sheikh-Hamad et al., 2000). Consistently, the incubation abolished the induction of Ostf mRNA expression. Since the change in intracellular ion strength would affect cell volume and cytoskeleton architecture (Di Ciano et al., 2002; Lionetto et al., 2002; Tse et al., 2006), we decided to test the effect of colchicine (an inhibitor of microtubule polymerization) to hypertonicity-induced Ostf expression. Obviously, colchicine treatment reduced Ostf gene expression. Although colchicine can inhibit spindle formation during mitosis, this effect on Ostf expression may not be significant in our study, as the time of drug treatment in the primary cell culture was relatively short (6 h). Therefore, our observation supports the notion that Ostf induction is dependent on the integrity of the cytoskeleton, which is modulated in the process of cell volume regulation. Using eel intestinal epithelia, Lionetto et al. reported that colchicine treatment inhibited the hypertonicity-induced short-circuit current by about 50–80% (Lionetto et al., 2002). This observation supports the important role of the cytoskeleton in cellular osmo-responses.

In summary, we are the first to measure the differential expression profiles of Ostf mRNA in gill CCs and PVCs during hyper- and hypo-osmotic adaptation. In addition, we have demonstrated the possible correlation between the expression of Ostf transcript and the two putative osmosensors (macromolecular crowding and cytoskeleton). The studies described here provide a fundamental understanding of the mechanisms of cellular osmoregulation in fish gills.

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REFERENCES

Chamberlin, M. E. and Strange, K. (1989). Anisosmotic cell volume regulation: a comparative view. *Am. J. Physiol.* **257**, C159-C173.

Choi, C. Y. and An, K. W. (2008). Cloning and expression of Na^+/K^+-ATPase and osmotic stress transcription factor 1 mRNA in black porgy, *Acanthopagrus schlegeli* during osmotic stress. *Comp. Biochem. Physiol.* **149B**, 91-100.

Di Ciano, C., Nie, Z., Szaszi, K., Lewis, A., Uruno, T., Zhan, X., Rotstein, O. D., Mak, A. and Kapus, A. (2002). Osmotic stress-induced remodeling of the cortical cytoskeleton. *Am. J. Physiol.* **283**, C850-C865.

Evans, D. H., Piermarini, P. M. and Choe, K. P. (2005). The multifunctional fish gill: dominant site of gas exchange, osmoregulation, acid-base regulation, and excretion of nitrogenous waste. *Physiol. Rev.* **85**, 97-177.

Fiol, D. F. and Kultz, D. (2005). Rapid hyperosmotic coinduction of two tilapia (*Oreochromis mossambicus*) transcription factors in gill cells. *Proc. Natl. Acad. Sci. USA* **102**, 927-932.

Fiol, D. F. and Kultz, D. (2007). Osmotic stress sensing and signaling in fishes. *FEBS J.* **274**, 5790-5798.

Fiol, D. F., Chan, S. Y. and Kultz, D. (2006). Regulation of osmotic stress transcription factor 1 (Ostf1) in tilapia (*Oreochromis mossambicus*) gill epithelium during salinity stress. *J. Exp. Biol.* **209**, 3257-3265.

Fiol, D. F., Mak, S. K. and Kultz, D. (2007). Specific TSC22 domain transcripts are hypertonically induced and alternatively spliced to protect mouse kidney cells during osmotic stress. *FEBS J.* **274**, 109-124.

Haussinger, D. (1996). The role of cellular hydration in the regulation of cell function. *Biochem. J.* **313**, 697-710.

Haussinger, D. (1998). Osmoregulation of liver cell function: signalling, osmolytes and cell heterogeneity. *Contrib. Nephrol.* **123**, 185-204.

Lang, F., Busch, G. L., Ritter, M., Volkl, H., Waldegger, S., Gulbins, E. and Haussinger, D. (1998a). Functional significance of cell volume regulatory mechanisms. *Physiol. Rev.* **78**, 247-306.

Lang, F., Busch, G. L. and Volkl, H. (1998b). The diversity of volume regulatory mechanisms. *Cell. Physiol. Biochem.* **8**, 1-45.

Lionetto, M. G., Pedersen, S. F., Hoffmann, E. K., Giordano, M. E. and Schettino, T. (2002). Roles of the cytoskeleton and of protein phosphorylation events in the osmotic stress response in eel intestinal epithelium. *Cell. Physiol. Biochem.* **12**, 163-178.

Sheikh-Hamad, D., Rouse, D. and Yang, Y. (2000). Regulation of stanniocalcin in MDCK cells by hypertonicity and extracellular calcium. *Am. J. Physiol.* **278**, F417-F424.

Strange, K. (2004). Cellular volume homeostasis. *Adv. Physiol. Educ.* **28**, 155-159.

Tse, W. K., Au, D. W. and Wong, C. K. (2006). Characterization of ion channel and transporter mRNA expressions in isolated gill chloride and pavement cells of seawater acclimating eels. *Biochem. Biophys. Res. Commun.* **346**, 1181-1190.

Tse, W. K., Au, D. W. and Wong, C. K. (2007). Effect of osmotic shrinkage and hormones on the expression of Na^+/H^+ exchanger-1, $Na^+/K^+/2Cl^-$ cotransporter and Na^+/K^+-ATPase in gill pavement cells of freshwater adapted Japanese eel, *Anguilla japonica*. *J. Exp. Biol.* **210**, 2113-2120.

Wehner, F., Olsen, H., Tinel, H., Kinne-Saffran, E. and Kinne, R. K. (2003). Cell volume regulation: osmolytes, osmolyte transport, and signal transduction. *Rev. Physiol. Biochem. Pharmacol.* **148**, 1-80.

Wong, C. K. and Chan, D. K. (1999). Isolation of viable cell types from the gill epithelium of Japanese eel *Anguilla japonica*. *Am. J. Physiol.* **276**, R363-R372.