Comparative Biochemistry and Physiology, Part A 162 (2012) 443–448

Contents lists available at SciVerse ScienceDirect

Comparative Biochemistry and Physiology, Part A

journal homepage: www.elsevier.com/locate/cbpa

Mitogen activated protein kinase 14-1 regulates serum glucocorticoid kinase 1 during seawater acclimation in Atlantic killifish, *Fundulus heteroclitus*

Emily G. Notch [a,b,c,*], Chris Chapline [c], Erin Flynn [c], Tess Lameyer [c], Alyson Lowell [c], Denry Sato [c], Joseph R. Shaw [c,d,e], Bruce A. Stanton [a,b,c]

[a] Department of Microbiology and Immunology, Dartmouth Medical School, Hanover, NH 03755, USA
[b] Department of Physiology, Dartmouth Medical School, Hanover, NH 03755, USA
[c] Mount Desert Island Biological Laboratory, Salisbury Cove, ME 04672, USA
[d] The School of Public and Environmental Affairs, Indiana University, Bloomington, IN 47405, USA
[e] The Center for Genomics and Bioinformatics, Indiana University, Bloomington, IN 47405, USA

ARTICLE INFO

Article history:
Received 19 December 2011
Received in revised form 28 February 2012
Accepted 25 April 2012
Available online 2 May 2012

Keywords:
MAPK14
SGK1
CFTR
Acclimation
Osmotic stress
Killifish

ABSTRACT

The Atlantic killifish (*Fundulus heteroclitus*) is an environmental sentinel organism used extensively for studies of environmental toxicants and osmoregulation. Previous research in our laboratory has shown that acute acclimation to seawater is mediated by an increase in SGK1. SGK1 promotes the trafficking of CFTR chloride channels from intracellular vesicles to the plasma membrane of the gill within the first hour in seawater resulting in increased chloride secretion. Although we have shown that the increase in gill SGK1 does not require activation of the glucocorticoid receptor, the mechanisms that mediate the rise SGK1 during acute acclimation is unknown. To test the hypothesis that mitogen activated protein kinase (MAPK14) is responsible for the rise in SGK1 we identified the coding sequence of killifish MAPK14-1 and designed a translational blocking vivo-morpholino targeting MAPK14-1. Injection of the MAPK14-1 vivo-morpholino resulted in a 30% reduction of MAPK14-1 and a 45% reduction in phosphorylated-MAPK14-1 protein in the gill of killifish transitioned from freshwater to seawater. Knock down of phosphorlyated-MAPK14-1 completely blocked the rise in SGK1 mRNA and protein in the killifish gill, providing the first direct and *in vivo* evidence that MAPK14-1 is necessary for acute seawater acclimation.

1. Introduction

The Atlantic killifish (*Fundulus heteroclitus*) is an environmental sentinel species commonly used for studies of salt balance (Hoffmann et al., 2002; Marshall, 2003; Scott et al., 2005; Fuller et al., 2007; Shaw et al., 2007; Hyndman and Evans, 2009; Flemmer et al., 2010; Whitehead, 2010). Killifish are euryhaline teleosts that inhabit estuaries and consequently are exposed to frequent and rapid alterations in salinity. Acclimation to seawater is mediated *via* increased Cl^- secretion through the cystic fibrosis transmembrane conductance regulator (CFTR) in mitochondria rich cells in the gills and opercular epithelium (Marshall, 2003). CFTR belongs to the ATP-binding cassette family of proteins, and mutations in the CFTR gene result in cystic fibrosis, the most common lethal autosomal recessive disorder in Caucasians (Guggino and Stanton, 2006).

The ability of killifish to acclimate from freshwater to seawater occurs in two stages. Long-term acclimation (>8 h in seawater) involves an increase in plasma cortisol, which activates the glucocorticoid receptor, leading to increases in CFTR mRNA and protein levels in the opercular membrane and gill (Shaw et al., 2007). Acute seawater acclimation (<8 h) is mediated by an increase in serum glucocorticoid kinase 1 (SGK1) mRNA and protein in the gill, which is necessary for trafficking of CFTR from intracellular vesicles to the plasma membrane resulting in increased Cl^- secretion (Sato et al., 2007; Shaw et al., 2008; Notch et al., 2011).

The mechanism responsible for the seawater induced increase in SGK1, which is required for acute acclimation in killifish, is unknown (Sato et al., 2007; Shaw et al., 2008; Notch et al., 2011). Previously, we demonstrated that the seawater induced increase in gill SGK1 is not mediated *via* increased plasma cortisol activation of the glucocorticoid receptor (Shaw et al., 2008). Several lines of evidence suggest that mitogen activated protein kinase 14 (MAPK14/p38MAPKα/SAPK2) may regulate the increase in SGK1 observed during acute acclimation to seawater. First, MAPK14 (stress activated protein kinase 2 (SAPK2)) is a stress response kinase that is induced by osmotic stress in a variety of organisms and cell types (Kültz et al., 1998; Bell et al., 2000; Kültz and Avila, 2001; Marshall et al., 2005; Chow and Wong, 2011). Second, mammalian SGK1 is transcriptionally regulated by MAPK14 (Firestone et al., 2003). Third, hyper-osmotic challenge increases phosphorylated-MAPK14

* Corresponding author at: Department of Microbiology and Immunology, Dartmouth Medical School, 604 Remsen Bldg, Hanover, NH 03755, USA. Tel.: +1 603 650 1534; fax: +1 603 650 1130.
E-mail address: emily.notch@dartmouth.edu (E.G. Notch).

1095-6433/$ – see front matter © 2012 Elsevier Inc. All rights reserved.
doi:10.1016/j.cbpa.2012.04.025

in cultured killifish opercular epithelia and cultured eel gill cells (Marshall et al., 2005; Chow and Wong, 2011). Collectively this evidence suggests, but does not prove the hypothesis that osmotic stress increases phosphorylated-MAPK14, which in turn activates SGK1. To test this hypothesis we obtained the full coding sequence of killifish MAPK14-1 and designed a translational blocking vivo-morpholino targeting MAPK14-1. Knock down of MAPK14-1 reduced total MAPK14-1 and phosphorlyated-MAPK14-1 protein levels and completely blocked the rise in SGK1 mRNA and protein in the killifish gill, providing the first evidence that MAPK14-1 is necessary for acute seawater acclimation.

2. Materials and methods

2.1. Adult killifish

All studies were performed in compliance with Institutional Animal Care and Use Committee guidelines approved by both the Mount Desert Island Biological Laboratory (10-01) and Dartmouth Medical School (10-03-03). *F. heteroclitus* were collected from Northeast Creek (Bar Harbor, ME) and held in aquaria containing running seawater at MDIBL (Salisbury Cove, ME, USA) or artificial seawater (27 ppt) at Dartmouth Medical School. At both sites, fish were acclimated to freshwater (0.3 ppt) by transitioning them to 10% seawater (3 ppt) for 2 weeks and then replacement of 10% seawater with "soft" freshwater (48 mg/L $NaHCO_3$, 30 mg/L $CaSO_4$, 30 mg/L $MgSO_4$, 2 mg/L KCl, pH 7.5–8.0) for 2 weeks prior to any experiments (ASTM, 1985; Marshall et al., 1999). Both male and female fish were used in this study. All fish utilized were sexually mature adults ranging in mass from 2 to 5 g. Results were similar in fish maintained at both sites; MDIBL and Dartmouth.

2.2. Cloning of killifish MAPK14-1 cDNA

To obtain sequence necessary to design a target-specific vivo-morpholino, cDNA containing the complete coding sequence of the killifish MAPK14-1 was cloned by PCR. First strand cDNA was synthesized from killifish liver total RNA (1 μg) with Taq polymerase and oligo(dT) primers (Promega, Madison, WI, USA). Unique oligonucleotide PCR primers were based on the known zebrafish MAPK14a cDNA sequence (NM_178223.2). Putative MAPK14 cDNA fragments were cloned into the pCR2.1 Topo-TA cloning vector (Invitrogen, Carlsbad, CA, USA), purified and sequenced. Nested MAPK14 cDNA fragments of 400 bp, 700 bp and 1 kb were identified. The 1 kb fragment generated by primers F4 (5′ GTTCTGCAGGCATGTCGCAGAAAG) and R3 (5′ CGGCTCTCAAAGCTCTGGTC) from two independent cDNA preparations corresponded to approximately 90% of the coding region of p38 MAPK in human, carp and zebrafish. 5′-RACE was performed with R8 primer (5′ TGTGGCAGGACTGAAAACATCTAAGAGGCC) using the SMART RACE PCR Kit (Clontech, Mountain View, CA, USA) to obtain the nucleotide sequence spanning the MAPK14-1 translation start site; 3′-RACE with F9 primer (5′CTCCAGCAAATAATGCGTCTGACAGGAACG) was performed to obtain the 3′-end of the MAPK14-1 coding sequence. Complete MAPK14-1 coding region amplicons were cloned into the pCR2.1 TOPO-TA vector (Invitrogen, Carlsbad, CA, USA) and sequenced. Alignment of killifish MAPK14-1 with other MAPK14 sequences was done using MacVector software (Mac Vector, Cary, NC, USA).

2.3. Vivo-morpholino injection

In a recent study we developed and reported on a novel vivo-morpholino technique to knock down genes in adult killifish (Notch et al., 2011). The translational blocking MAPK14-1 vivo-morpholino (5′CCGGATCTTTCTTTCTGCGACATTC) was designed to be complimentary to the putative initiation codon of *F. heteroclitus* MAPK14-1 sequence obtained by RACE, as described above. Freshwater acclimated adult killifish were intraperitoneal (IP) injected with 15 μg/g MAPK14-1 translational blocking vivo-morpholino, or an appropriate concentration of control vivo-morpholino (5′ CCTCCTACCTCAGTTCCAATTTATA). Fish were returned to freshwater for 4 h with temperature control (13–15 °C) and aeration. After 4 h in freshwater, fish were transferred to seawater for 1 h, except for freshwater control vivo-morpholino treated fish that were transferred to another tank of freshwater for 1 h.

2.4. Western blotting

Fish were sacrificed by cervical dislocation, and gills surgically removed and homogenized according to standard protocols in lysis buffer (25 mM Hepes, 10% v/v glycerol, and 1% v/v Triton-X with Complete Protease Inhibitor (Roche Applied Science, Indianapolis, IN, USA) and Halt phosphatase inhibitor (Pierce, Rockford, IL, USA)) for western blotting (Chow and Wong, 2011; Firestone et al., 2003). Samples were manually homogenized and centrifuged at 14,000 × g for 10 min in a refrigerated centrifuge. Protein samples were combined with an equal volume of Laemmli buffer with 1 mM DTT and heat denatured at 85 °C for 5 min. Samples were stored at −20 °C. Western blot analysis of MAPK14 (anti-MAPK14 318–330, #M1822, 1:1000 dilution, Sigma-Aldrich, St. Louis, MO), SGK1 (anti-SGK #S5188, 1:2000 dilution, Sigma-Aldrich, St. Louis, MO, USA), Na^+, K^+–ATPase (Na^+, K^+–ATPase a5 supernatant, 1 μg/mL, Developmental Studies Hybridoma Bank, University of Iowa, Iowa City, IA, USA), and β-actin (Clone C4, 1:2000 dilution, MP Biomedicals, Solon, OH, USA) in gill lysates was performed as previously described (Shaw et al., 2007, 2008). Phosphorylated MAPK14 was detected with a commercially available antibody (phospho-p38 MAP Kinase (Thr180/Tyr182), 1:2000 dilution, Cell Signaling, Beverly, MA, USA) previous used with killifish samples (Marshall et al., 2005). Band intensity was analyzed with Image J software (NIH). Pixel intensity of individual bands was normalized to β-actin to account for any loading difference per well. Entire blots were adjusted for brightness and contrast levels using Adobe Photoshop CS4 (Adobe, San Jose, CA, USA).

2.5. MAPK14 antibody verification

To demonstrate that the MAPK14 antibody recognizes killifish MAPK14-1, the full cDNA sequence of killifish MAPK14-1 was cloned into a pcDNA3.1 (Invitrogen, Carlsbad, CA, USA) expression vector where a CMV promoter drives expression. The pcDNA-kf MAPK14-1 construct was sequenced to ensure correct orientation, and appropriate start and stop codons. pcDNA-kf MAPK14-1 was transiently transfected into HEK-293 cells with Effectene transfection reagent (Qiagen, Valencia, CA, USA). Cells were seeded in 6 well plates at 2×10^6 cells per well and allowed to adhere for 24 h prior to transfection. Cells were transfected with 200 ng, 400 ng, 800 ng, or 1200 ng of pcDNA-kf MAPK14-1, or 1200 ng empty pcDNA3.1 vector (mock). Cells were harvested 24 h after transfection. Briefly, cells were rinsed three times with phosphate buffered saline with 1 mM $MgCl_2$ and 0.1 mM $CaCl_2$ and subsequently lysed with lysis buffer (25 mM Hepes, 10% v/v glycerol, and 1% v/v Triton-X with Complete Protease Inhibitor (Roche Applied Science, Indianapolis, IN, USA)) was added to each well. Samples were manually homogenized and centrifuged at 14,000 × g for 10 min in a refrigerated centrifuge. Protein samples were combined with an equal volume of Laemmli buffer with DTT (1 mM) and heat denatured at 85 °C for 5 min. Samples were stored at −20 °C until time of analysis.

2.6. Quantitative PCR

Fish were sacrificed by cervical dislocation, and gills were excised, rinsed and placed in RNAlater (Ambion, Austin, TX, USA) until time of