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Mitogen Activated Protein Kinase 14-1 Regulates Serum Glucocorticoid Kinase 1 During Seawater Acclimation in Atlantic Killifish, *Fundulus heteroclitus*

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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 show 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 phosphorylated-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.

Keywords

MAPK14; SGK1; CFTR; acclimation; osmotic stress; killifish

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;

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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 (>8h 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; Stanton 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/p38MAPK α /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 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 phosphorylated-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). *Fundulus 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 2–5 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' CCTCTACCTCAGTTCCAATTTATA). 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 [4,5]. 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 1mM 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; Shaw et al., 2008). Phosphorylated MAPK14 was detected with a commercially available antibody (Phospho-p38 MAP Kinase (Thr180/Tyr182), 1:2000 dilution, Cell Signaling, Beverly, MA, USA) previously 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 (1mM) 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 use. RNA was isolated using the RNeasy kit (Qiagen). Briefly, gill samples were thoroughly homogenized with a Tissue-Tearor (Biospec Products, Bartlesville, OK, USA) in lysis buffer containing guanidine-thiocyanate and then mixed with an equal volume of ethanol. Samples were immediately bound to a glass fiber filter and washed three times prior to elution with nuclease free water. Samples were immediately DNase treated (DNAfree, Ambion). RNA integrity and concentration was assessed using micro-capillary electrophoresis on an Agilent 2100 Bioanalyzer. RNA was compared to a RNA ladder with 6 RNA transcripts of varying sizes and known concentration of 150 ng/ μ L. RNA quality was verified by observation of corresponding 18S and 28S peaks on the electropherogram. Only intact RNA was used for further analysis.

For quantitative PCR (RT-qPCR) of SGK1 cDNA was synthesized from 1 μ g of total RNA using Retroscript Reverse Transcriptase (Ambion) with random decamers. For fluorescence based RT-qPCR reactions containing Sure start Taq Polymerase (Stratagene, Santa Clara, CA, USA), SYBR green (Stratagene Brilliant SYBR), 300 nM Reference dye, 2.5 mM $MgCl_2$, 100 ng cDNA, 36–60 nM primers and nuclease free water were amplified with initial denaturing at 95 °C for 10 min, followed by 40 cycles of 30 s at 95 °C and 1 min at 55 °C. SGK1 specific primers were designed and validated as previously described (Notch et al., 2011). Efficiencies of all reactions were examined prior to analysis. Transcript abundance was calculated as copy number, based on serial dilution of a plasmid standard curve containing the entire killifish SGK1 sequence (pcDNA3.1-kf SGK1). Standard curves of copy number plotted against threshold cycle (Ct) were linear over a 6-log range, and had a correlation coefficient close to 1 ($R^2 = 0.99$).

2.7 Statistics

Data presented are mean $\pm$ SEM. Statistical significance was determined by one-way ANOVA followed by a Tukey's HSD post-hoc test using a p value <0.05 as significant. Statistical analysis was performed with Prism v 5.0 (Graph Pad Software, San Diego, CA, USA).

3. Results

3.1 Killifish MAPK14-1

Killifish MAPK14-1 sequence was obtained through PCR amplification and 5' - and 3' - RACE. The full length coding sequence of killifish MAPK14-1 is 1074 nucleotides, translating to a 357 amino acid protein (Figure 1). The full length killifish (*Fundulus heteroclitus*) MAPK14-1 protein shares 89% amino acid sequence identity with zebrafish (*Danio rerio*) MAPK14a ([NP_571797.1](#)), 86% identity with human (*Homo sapiens*) MAPK14 ([CAG38743](#)) and 82% sequence identity with African clawed frog (*Xenopus laevis*) MAPK14 ([NP_001080300.1](#)).

Before conducting western blots studies to determine the efficacy of MAPK14-1 vivo-morpholino knock down, we first verified that a commercially available MAPK14 antibody from Sigma recognized killifish MAPK14-1. Full-length killifish MAPK14-1 cDNA was expressed in HEK293 cells and MAPK14 protein levels were measured by western blot. A dose responsive increase in MAPK14-1 protein levels corresponded with increased cDNA concentration demonstrating that the antibody recognized killifish MAPK14-1 (Figure 2). Background levels of MAPK14-1 were detectable in HEK293 cells (Figure 2A lane labeled "mock") as the antibody used was designed to detect human MAPK14.

3.2 Knock down of gill MAPK14-1 and phosphorylated-MAPK14-1

Freshwater acclimated killifish were IP injected with control vivo-morpholino, or an equal concentration of MAPK14-1 translational blocking vivo-morpholino. After 4h in freshwater, fish were transferred to seawater for 1h, except for freshwater control vivo-morpholino treated fish that were transferred to another tank of freshwater for 1h. Transfer from freshwater to seawater did not increase MAPK14-1 protein levels in fish injected with the control morpholino. However, the MAPK14-1 vivo-morpholino significantly reduced, by 30%, gill MAPK14-1 protein levels in fish transferred from freshwater to seawater, compared to similarly transitioned fish injected with the control vivo morpholino (Figure 3A and 3B). Fish injected with MAPK14-1 vivo-morpholino remaining in freshwater for up to 24 h post-injection showed no significant changes in gill MAPK14-1 protein levels (103% of Control).

Transfer from freshwater to seawater (in fish injected with the control vivo-morpholino) increased phosphorylated MAPK14 protein levels. The MAPK14-1 vivo-morpholino significantly ($p<0.05$) reduced (46%) the amount of phosphorylated-MAPK14 protein in gill of fish transitioned from freshwater to seawater in comparison to fish injected with equal concentrations of control vivo-morpholino (Figure 3C and 3D).

3.3 Knock down of MAPK14 blocks the seawater induced increase in SGK1 mRNA and protein levels, but does not alter Na⁺,K⁺-ATPase protein levels

Transition from freshwater to seawater resulted in a 1.7 fold increase in gill SGK1 mRNA and a 1.8-fold increase in SGK1 protein in comparison to fish remaining in freshwater (Figure 4), consistent with previously published studies (Shaw et al., 2008; Notch et al., 2011). Fish injected with the MAPK14-1 vivo-morpholino and transitioned from freshwater to seawater had significantly ($p<0.05$) lower gill SGK1 mRNA levels in comparison to fish

injected with an equal concentration of control vivo-morpholino and transitioned to seawater. The MAPK14-1 vivo-morpholino also reduced gill SGK1 protein by 57% ($p<0.05$) in comparison to the control morpholino in fish transitioned from freshwater to seawater (Figure 4).

To validate that the MAPK14-1 morpholino did not have off target effects on other proteins important for fresh to seawater acclimation, Na^+,K^+ -ATPase protein levels in the gill were examined. No difference in Na^+,K^+ -ATPase gill protein levels were found between any of the treatment groups (Figure 5).

4. Discussion

This study provides the first direct evidence that upon transition from freshwater to seawater an increase in phosphorylated-MAPK14-1 is responsible for the rise in SGK1 mRNA and protein in acute seawater acclimation. This combined with previous studies that show SGK1 in turn mediates the acute increase in plasma membrane CFTR in gill of killifish, a response that is an important component of the acclimation response to a seawater challenge, provide a more complete model of the initial steps necessary for acute acclimation (Notch et al., 2011). The results of this paper are consistent with other publications demonstrating that transfer from freshwater to seawater increases the amount of phosphorylated MAPK14-1 but not the amount of total MAPK in killifish gill and opercular membrane (Kültz and Avila, 2001; Marshall et al., 2005). Studies in cultured eel gill cells have also shown that hyper-osmotic stress increases phosphorylated-MAPK14 levels (Chow and Wong, 2011). It is important to note, however, that this is the first study to show *in vivo* that an increase in the amount of phosphorylated-MAPK14 increases SGK1 mRNA in teleost gills. In mammals, phosphorylated-MAPK14 transcriptionally activates SGK1 (Bell et al., 2000; Firestone et al., 2003) and in cultured eel gill cells an increase in phosphorylated-MAPK14 occurs within minutes of hyper-osmotic stress (Chow and Wong, 2011). This rapid response in SGK1 is consistent with previous studies showing that the rise in SGK1 mRNA occurs as early as 15 min after transition to seawater (Shaw et al., 2008). One interesting finding from this study is the result that knock-down of MAPK14-1 significantly reduces levels of phosphorylated-MAPK14-1 in the gill upon transition to seawater. Since MAPK14 has been shown to autophosphorylate in several species including humans, mice and Atlantic salmon, it is likely that the reduction in total MAPK14-1 we observe with knock down is sufficient to reduce the amount of autophosphorylated MAPK14-1 (Kim et al., 2005; Askari et al., 2009; Rothweiler et al., 2011). This is an important finding of the study, as phosphorylated MAPK14-1 is necessary for downstream signaling (Kang et al., 2006; Pimienta and Pascual, 2007).

Collectively, the current study and previous reports by our laboratory and others provide a more complete understanding of acute seawater acclimation in killifish. These data reveal a model in which transfer from freshwater to seawater increases the amount of phosphorylated MAPK14, which transcriptionally activates SGK1 leading to CFTR trafficking from an intracellular compartment to the plasma membrane that in turn is responsible for enhanced Cl^- secretion and acclimation to seawater (Shaw et al., 2008; Notch et al., 2011). This study is an example of the utility of the vivo morpholino approach in adult killifish, developed by our laboratory (Notch et al., 2011), to directly examine the role of genes in the homeostatic response to an environments stressor.

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Figure 1.

Alignment of the amino acid sequences of MAPK14-1 from *Fundulus heteroclitus*, *Danio rerio*, *Homo sapiens* and *Xenopus laevis*. Bold denotes identical residues.

Figure 2.

Demonstration that the MAPK14 antibody recognized killifish MAPK14-1. Different concentrations of a plasmid containing cDNA of the complete coding sequence of killifish MAPK14-1 were transfected into HEK-293 cells. **A.** Representative western blot of MAPK14, **B.** Summary of MAPK14-1 western blot experiments. N=3/group.

Figure 3.

MAPK14-1 and phosphorylated MAPK14-1 protein levels in gills of killifish injected with a control vivo-morpholino or a translational blocking vivo-morpholino targeting MAPK14-1.

A. Representative western blot of MAPK14-1. **B.** Summary of MAPK14-1 western blot experiments. **C.** Representative western blot of phosphorylated-MAPK14-1. **D.** Summary of phosphorylated-MAPK14-1 western blot experiments. Freshwater acclimated killifish were injected with control vivo-morpholino or MAPK14-1 vivo-morpholino. FW-C: Freshwater control vivo-morpholino, FSW-C: Freshwater to seawater control vivo-morpholino, FSW-M: Freshwater to seawater MAPK14-1 vivo-morpholino. N=9/group. Different letters indicated statistically significant differences between groups $p < 0.05$.

Figure 4.

SGK1 mRNA and protein levels in gills of killifish injected with control vivo-morpholino or translational blocking vivo-morpholino targeting MAPK14-1. **A:** SGK1 mRNA abundance. **B:** Representative western blot of SGK1. **C:** Summary of SGK1 western blot experiments. Freshwater acclimated killifish were injected with control vivo-morpholino or MAPK14-1 vivo-morpholino. FW-C: Freshwater control vivo-morpholino, FSW-C: Freshwater to seawater control vivo-morpholino, FSW-M: Freshwater to seawater MAPK14-1 vivo-morpholino. N=9/group. Different letters indicated statistically significant differences between groups $p < 0.05$.

Figure 5.

Na⁺,K⁺-ATPase protein levels in gills of killifish injected with control vivo-morpholino or translational blocking vivo-morpholino targeting MAPK14-1. **A:** Representative western blot of Na⁺,K⁺-ATPase. **B:** Summary of Na⁺,K⁺-ATPase western blot experiments. Freshwater acclimated killifish were injected with control vivo-morpholino or MAPK14-1 vivo-morpholino. FW-C: Freshwater control vivo-morpholino, FSW-C: Freshwater to seawater control vivo-morpholino, FSW-M: Freshwater to seawater MAPK14-1 vivo-morpholino. N=9/group. Different letters indicated statistically significant differences between groups $p < 0.05$.