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A novel GRAIL E3 ubiquitin ligase promotes environmental salinity tolerance in euryhaline tilapia

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ABSTRACT

Background: Tilapia (*Oreochromis mossambicus*) are euryhaline fishes capable of tolerating large salinity changes. In a previous study aimed to identify genes involved in osmotolerance, we isolated an mRNA sequence with similarity to GRAIL (Gene Related to Anergy In Lymphocytes), which is a critical regulator of adaptive immunity and development. Tilapia GRAIL contains a PA (protease associated) domain and a C3H2C3 RING finger domain indicative of E3 ubiquitin ligase activity.
Scope of review: Western blots analysis was used to assess GRAIL expression pattern and responses to hyperosmotic stress. Immunohistochemistry was used to reveal the cellular localization of GRAIL in gill epithelium. Overexpression in HEK293 T-Rex cells was used to functionally characterize tilapia GRAIL. Salinity stress causes strong up-regulation of both mRNA and protein levels of tilapia GRAIL in gill epithelium. Tissue distribution of GRAIL protein is mainly confined to gill epithelium, which is the primary tissue responsible for osmoregulation of teleost fishes. Overexpression of tilapia GRAIL in HEK293 cells increases cell survival (cell viability) while decreases apoptosis during salinity challenge.
Major conclusions: Our data indicate that tilapia GRAIL is a novel E3 ubiquitin ligase involved in osmotic stress signaling, which promotes environmental salinity tolerance by supporting gill cell function during hyperosmotic stress.
General significance: Involvement of tilapia GRAIL in the osmotic stress response suggests that GRAIL E3 ubiquitin ligases play a broader role in environmental stress responses, beyond their documented functions in adaptive immunity and development.

1. Introduction

The global mean sea level of the oceans has been rising and is projected to continue to rise at an accelerated rate due to global climate change, which will induce salinization of coastal freshwater habitats, in particular in low-lying river deltas, e.g., in Southeast Asia [1,2]. In addition, increased evaporation and droughts resulting from global warming are projected to cause salinity increases in inland water systems [2]. Because coastal areas are rich in life, both from a species diversity and a biomass perspective, salinization of those areas will significantly impact ecosystem structure. Understanding how organisms respond to salinity stress and how evolution has equipped some organisms (euryhaline species) with the ability to tolerate large increases in salinity sheds light on biological processes and molecular functions that will be subject to increased selection pressure in coastal ecosystems in the future.

Tilapia (*Oreochromis mossambicus*) is a euryhaline species of bony fish (teleost) that is native to the Great East African rift lakes but has invaded many marine and limnic ecosystems world-wide. This fish represents an excellent model for studying salinity tolerance because it utilizes the underlying mechanisms very effectively to gain an extreme salinity tolerance range of 0 (fresh water, FW) to 120 ppt (>3× seawater, SW) [3]. Tilapia and other euryhaline fishes are capable of switching the direction of active ion transport across their gill epithelium from salt absorption mode (in plasma–hyposmotic environments such as FW) to salt secretion mode (in plasma–hyperosmotic environments such as SW). Such switching entails many molecular and morphological adjustments that lead to reorganization of the gill epithelium and is controlled by an osmotic stress signaling network [4]. While characterizing this network, we have recently identified a gene that is highly and rapidly induced during salinity stress in tilapia gill epithelium and shows a high degree of similarity to Gene Related to Anergy In Lymphocytes (GRAIL) E3 ubiquitin ligases [5].

The original GRAIL prototype was discovered in mice based on its induction paralleling the occurrence of an anergic phenotype in CD4(+) CD25(−) T cells [6]. A dominant negative mutant of GRAIL suppressed

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the anergic phenotype suggesting that GRAIL is required for T cell anergy, which is a state of immune unresponsiveness resulting from stimulation of T cell antigen receptor [7]. GRAIL is a transmembrane E3 ubiquitin ligase with homology to RING zinc-finger proteins [8]. An alternative name for GRAIL that has been used for *Drosophila*, ascidian, human, and rat GRAIL orthologs is Goliath [9–12]. *Drosophila* Goliath was discovered prior to mouse GRAIL and has a role in development [10]. GREUL1 (Goliath Related E3 Ubiquitin Ligase 1) has been identified in *Xenopus*, where it is also involved in development [13].

E3 ubiquitin ligases are key players in the process of protein ubiquitination, which represents a posttranslational modification that alters many proteins either by mono- or polyubiquitination. Ubiquitination is carried out by the sequential action of several proteins including E1 (ubiquitin activating), E2 (ubiquitin conjugating), and E3 (ubiquitin ligases) enzymes. The specificity of the reaction relies on the action of the E3 ubiquitin ligases, many hundreds of which are encoded by animal genomes. Each E3 enzyme has typically a narrow group of targets (substrate proteins). Functional consequences of ubiquitination are diverse and depend on ubiquitin chain length and topology. They include proteasome-mediated degradation, protein sorting, protein kinase activation and inactivation, DNA repair, cell cycle regulation, and apoptosis [14].

The present study analyzes the structure and functions of a new E3 ubiquitin ligase—tilapia GRAIL, its localization, and its regulation during exposure of tilapia to environmental salinity stress. Our data indicate that tilapia GRAIL participates in osmotic stress signaling and promotes environmental salinity tolerance by supporting gill cell function and survival and by suppressing apoptosis during hyperosmotic stress.

2. Materials and methods

2.1. Animals

Tilapia (*O. mossambicus*) were maintained in large (4-ft. diameter) tanks supplied with flow-through heated (26 °C) well water (=FW, Na^+=28 mg l^{-1}, K^+<5 mg l^{-1}, Ca^{2+}=33 mg l^{-1}, Mg^{2+}= 36 mg l^{-1}, pH 8.0) at the Center for Aquatic Biology and Aquaculture (CABA) of the University of California, Davis. SW acclimated fish were maintained at least for 4 weeks in SW tanks. SW was collected at Bodega Bay, California (1000 mOsm/kg, ~32 ppt) or prepared with Instant Ocean sea salt to the same concentration. Fish were sampled, gills were perfused, and gill epithelium was collected by scraping off from the cartilage of individual gill arches as described in previous work [15]. In addition, muscle (posterior to the dorsal fin), and the whole intestine, kidneys, heart, brain, liver, and testis or ovaries were collected. All different tissues were kept at −80 °C and subsequently utilized for RNA or protein isolation. All procedures were approved by the University of California, Davis Institutional Animal Care and Use Committee (IACUC, Protocol #15013).

2.2. Cloning of full-length tilapia GRAIL

Full-length sequence for tilapia *GRAIL* gene was cloned using SMART RACE cDNA Amplification kit (Clontech, USA). Tilapia *GRAIL* clone SSH#30 was used as a starting point and extended with 3′ RACE and 5′ RACE until completion of the full-length cDNA. Sequence was submitted to Genbank (DQ465380).

2.3. Protein extraction and Western blot analysis

For protein extraction, cells were lysed in a buffer that contained 50 mM Tris–HCl, pH 7.4, 1% Nonidet P-40, 0.25% sodium deoxycholate, 150 mM NaCl, 1 mM EDTA, 1 tablet of minicomplete protease inhibitor mixture (Roche) per 10 ml, 1 mM activated Na_3VO_4, and 1 mM NaF. Protein concentrations were determined by BCA protein assay according to the manufacturer's instructions (Pierce). Protein separation by SDS-PAGE (25 μg/lane) and Western blots were carried out as described previously [16]. A custom-made anti-tilapia GRAIL antibody at 1:500 dilution was used (raised in rabbits by Sigma-Genosys using as antigen the peptide DDRKESFSAESPPD, amino acid 303 to 316). Blots were developed with SuperSignal Femto (Pierce) and imaged with a ChemiImager (Alpha Innotech).

2.4. Immunohistochemistry

Branchial tissues were fixed and embedded in paraffin using a Tissue Tek vacuum infiltration processor (Sakura Finetek, Torrance, CA, USA). Paraffin blocks were constructed with a Tissue Tek tissue embedding center (Sakura Finetek, Torrance, CA, USA). Blocks were sectioned at 5-μm thickness using a Bromma 2218 Historange microtome (LKB, Uppsala, Sweden) and sections were floated on to a poly-lysine-coated glass microscope slide; slides were dried overnight at 44 °C. Once dry, slides were deparaffinized in xylene for 5 min (3×), 100% EtOH (2×), 95% EtOH (2×), and 80% EtOH (1×). For detection, slides were incubated in blocking solution (PBS containing 2% BSA (Roche Applied Science)) for 1 h at room temperature. After blocking, slides were incubated with anti-tilapia GRAIL antibody; 1:80 in blocking solution for 1 h at room temperature. Slides were washed three times for 5 min each in PBS and then incubated with Alexa Fluor® goat anti-rabbit IgG antibody (A-11070, Molecular Probes, Eugene, OR, USA) diluted 1:100 in PBS containing 2% BSA for 1 h at room temperature. After five washes in PBS, slides were counterstained in propidium iodide (PI) (Sigma; 10 μg/ml) for 5 min at room temperature. After three washes for 5 min in PBS, slides were dried, 20 μl of ProLong® Gold antifade reagent (P36930, Invitrogen) was applied, and coverslips were mounted and sealed with clear nail polish. Slides were examined with a laser scanning cytometer (Compucyte, Cambridge, MA, USA) using a 40× objective (UPlanFL 40×/0.75/∞/0.17, Olympus, Melville, NY, USA) in combination with UV and Argon lasers (400, 488 nm). Images were acquired using Wincyte software (Compucyte). Controls using preimmune rabbit IgG instead of primary antibody were processed in parallel and were negative.

2.5. Overexpression of tilapia GRAIL in HEK293 T-Rex cells

Tilapia GRAIL ORF was amplified with primers ATGGGTGAGAAGACACAACCAC and TCATCCCCTTGCAGCCTCTGAG. Amplified product was confirmed by double-pass sequencing. Construct pcDNA5/GRAIL was created by inserting PCR product into pcDNA5/FRT/TO TOPO TA expression vector (Invitrogen). The construct was then propagated in *Escherichia coli* strain DH5α (Invitrogen). Endotoxin-free plasmid Megapreps were performed using a kit as described by the manufacturer (Qiagen GmbH, Hilden, Germany). Stable cell lines were established by transfecting HEK293 T-Rex cells (Invitrogen) with 2 μg of a 1:9 mix of pcDNA/GRAIL plasmid DNA:pOG44 plasmid DNA and 4 μl of LipofectAMINE 2000 reagent (Invitrogen). Twenty-four hours after transfection, cells were exposed to selection in medium containing 0.15 μg/ml hygromycin (Invitrogen). After 2 weeks, individual colonies were picked, expanded, and tested for expression of tilapia GRAIL and qPCR analysis. In parallel, HEK293 T-Rex cells were transfected with empty pcDNA5/FRT/TO TOPO TA expression vector in order to obtain the Empty cell line used as control in the experiments.

2.6. Cell viability and apoptosis assays

HEK293 cells were cultured in white-walled 96-well plates and exposed to isoosmotic (300 mOsm/kg) or variable hypersaline (500, 550, 575, 600, 625, 650, 675, 725, 757, and 830 mOsm/kg) media. Hypersaline media were prepared by addition of an appropriate amount of NaCl to isosmotic medium and osmolality confirmed after thorough mixing using a freezing point osmometer (μOsmette, Advanced Instruments,