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Zebrafish Plzf transcription factors enhance early type I IFN response induced by two non-enveloped RNA viruses

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ABSTRACT

The BTB-POZ transcription factor Promyelocytic Leukemia Zinc Finger (PLZF, or ZBTB16) has been recently identified as a major factor regulating the induction of a subset of Interferon stimulated genes in human and mouse. We show that the two co-orthologues of PLZF found in zebrafish show distinct expression patterns, especially in larvae. Although *zbtb16a/plzfa* and *zbtb16b/plzfb* are not modulated by IFN produced during viral infection, their over-expression increases the level of the early type I IFN response, at a critical phase in the race between the virus and the host response. The effect of Plzfb on IFN induction was also detectable after cell infection by different non-enveloped RNA viruses, but not after infection by the rhabdovirus SVCV. Our findings indicate that *plzf* implication in the regulation of type I IFN responses is conserved across vertebrates, but at multiple levels of the pathway and through different mechanisms.

1. Introduction

In vertebrates, antiviral innate immunity is primarily based on the stimulation of type I IFN pathway. After virus recognition, Pattern Recognition Receptors (PRR) trigger signalling pathways leading to the induction of type I IFNs. These cytokines are secreted, and, when bound on surface IFN receptors, promote via the Jak/STAT pathway the transcription of a large number of Interferon Stimulated Genes (ISGs), some of which have antiviral activity.

Teleost fish possess typical type I IFNs, which mediate potent antiviral activities as IFNα and IFNβ do in mammals (reviewed in (Langevin et al., 2013a)). Fish PRR specialized in virus recognition comprise RIG-I related receptors and Toll-like receptors, some of which are specific to fish - such as TLR22 that recognizes viral RNAs. Signalling pathways triggered by these sensors involve orthologues of key kinases and transcription factors of IRF or NFkB pathways, including TBK1, IRF3 and IRF7. Regarding the effectors of antiviral immunity, a core set of ISGs is conserved between fish and mammals, whereas multigenic families comprising ISGs have often diversified independently during fish and tetrapod evolution (Briolat et al., 2014). Many ISGs involved in the antiviral signalling are also highly conserved, denoting that the main feedback loops of the IFN pathway are similar in fish and mammals. Although canonical signalling pathways of the IFN antiviral axis are indeed well conserved across vertebrates, the implication of recently discovered mediators of these pathways often remains uncertain in fish.

The transcription factor Promyelocytic Leukemia Zinc Finger (PLZF) – aka ZBTB16 -is one of such recently discovered regulators of type I IFN system in men and mice. In a seminal study, Xu et al. found that in the presence of IFN, PLZF associates with Histone Deacetylase (HDAC)-1 and the TRIM protein PML to up-regulate the expression of an important subset of ISGs (Xu et al., 2008). PLZF is a member of the Broad-complex, Tramtrack, Bric-à-Brac – Poxvirus and Zinc finger (BTB-POZ) family of transcriptional regulators, which is characterized by a N-terminal BTB domain and C-terminal Zinc-fingers repeats connected by a hinge region. These transcription factors have been implicated in many processes such as development, germ cell and leukocyte differentiation, and cell cycle regulation (reviewed in (Siggs and Beutler, 2012)). The biological functions of BTB-POZ proteins are most frequently associated with their roles as transcriptional repressors: upon post translational modifications (Ball et al., 1999), (Costoya et al., 2008), (Kang et al., 2003), (Chao et al., 2007), (Nanba et al., 2003) BTB-POZ proteins can recruit co-repressors (Huynh and Bardwell, 1998; Melnick et al., 2000), histone deacetylases (Costoya et al., 2008) (Rui et al., 2012)

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and ubiquitin-ligases (Mathew et al., 2012), to build complexes that typically modify chromatin conformation and modulate gene expression. However, BTB-POZ proteins can also activate transcription as shown for PLZF with ISGs during the antiviral response. In mammals, PLZF directly regulated the promoters of targeted ISGs and overexpression of PLZF activated *rsad2*-Luciferase reporter. This activity is greatly enhanced by co-expression of HDAC1 and by the presence of type I IFN that triggers PLZF phosphorylation at positions that are critical for its role as a transcriptional inducer. As *plzf*-KO mice showed a severe defect in ISG induction and a higher susceptibility to different RNA viruses, this gene plays an important role in antiviral defenses *in vivo* and does not represent a secondary redundant level of regulation (Xu et al., 2008).

PLZF is conserved across vertebrates, but most fish species appear to possess two paralogues. To date, most studies of fish Plzf have focused on Plzfa as a marker of male germ cells (Ozaki et al., 2011) (Kawasaki et al., 2012) (Wong and Collodi, 2013), where it may play a similar role as in mammals where it is involved in spermatogonial stem cell self-renewal (Buaas et al., 2004) (Costoya et al., 2004). The degradation of Plzf also mediates a feedback loop that allows neuronal progenitors to undergo differentiation in zebrafish (Sobieszczuk et al., 2010). Altogether these reports indicate that the repressor activity of PLZF contributes to the maintenance of progenitors of diverse cell lineages in fish and mammals.

In this work, we show that besides these functions, fish Plzf proteins are also involved in the activation of type I IFN response, enhancing the expression of Ifnφ1 itself during the critical early stages of responses to viruses and poly I:C.

2. Materials and methods

2.1. Ethics statement

All animals were handled in strict accordance with good animal practice as defined by the European Union guidelines for the handling of laboratory animals (http://ec.europa.eu/environment/chemicals/lab_animals/home_en.htm) and by the Regional Paris South Ethics committee. Experimental protocols involving zebrafish were approved by the INRA institutional ethical committee "Comethea" (#12/114). All animal work was approved by the Direction of the Veterinary Services of Versailles (authorization number 78–28) as well as INRA (authorization number B78-720) or Pasteur institute (B75-15-22) fish facilities.

2.2. Fish

Wild-type AB, initially purchased from the ZIRC (Zebrafish International Resource Center, Eugene, OR), were raised in our fish facility. All staging in the text refers to the standard 28,5 C developmental time. Larvae were anesthetized with 200 μg/ml tricaine (Sigma–Aldrich). Adult fish were sacrificed by lethal anesthesia with eugenol (0.2% clove essential oil). Chikungunya infections of zebrafish larvae were performed as described in (Palha et al., 2013).

2.3. Whole mount in situ hybridization

RNA probes were designed to cover the hinge-coding region. Templates for RNA probe synthesis were PCR-amplified from cDNA (3dpf larvae) using following primers for *plzfa*: Plzfa ISH Fw and Plzfa ISH Rev (product size 717bp), and for *plzfb*: Plzfb ISH Fw and Plzfb ISH Rev (product size 805bp) (Table 1). Primers were removed with Illustra MicroSpin S-400 HR column (GE Healthcare). Antisense RNA probes were synthesized with T3 polymerase (Promega) in the presence of digoxigenin-11-UTP (Roche Applied Science) and purified with NucAway spin column (Ambion). Whole-mount *in situ* hybridization was done as in (Thisse and Thisse, 2008) with a hybridization temperature of 55 °C and using NBT/BCIP relevation (Sigma).

2.4. Immunocytochemistry

Cells overexpressing PLZF were fixed in 4% PFA/PBS for 20 min at 4 °C. Cells were then permeabilized in PBS/0.2% TritonX100 for 5 min at RT and before saturation in 2% BSA/PBS solution at RT for 1hr. Cells were then incubated with anti-HA (Roche) monoclonal antibody in 2%BSA/0.1% TritonX100/PBS for 1 h prior to incubation with an anti-mouse secondary antibody coupled to Alexa 594 (Molecular Probes) in 2% BSA/0.1% Triton X100/DAPI/PBS for 1 h at RT before mounting in Immuno Mount solution (Molecular Probes). Images were acquired on AxioObserver Z1 microscope (Zeiss) with a 63x Plan Neofluar objective using Photometrics CoolSNAP HQ2 Camera.

2.5. Plasmids

Zebrafish *plzfa* and *plzfb* open reading frames were amplified from cDNA prepared from 3 days post-fertilization whole zebrafish larvae respectively using Plzfa Fw and Rev primers and PlzfbFw and Rev primers (Table 1). Amplification product were cloned with a HA-tag into pcDNA3.3 expression vector using pcDNA™3.3-TOPO® TA Cloning® Kit (ThermoFisher). Similarly, Plzfb deletion mutant was synthesized by amplification of the C-terminal end comprising the last eight zinc-finger repeats, before cloning into the pcDNA3.3 expression vector.

2.6. Cells and viruses

Epithelioma papulosum cyprini (EPC) (ATCC CRL2872) cell line was maintained in Glasgow's modified Eagle's medium-HEPES 25 mM medium (Eurobio) supplemented with 10% fetal bovine serum (FBS, Eurobio), 1% tryptose phosphate broth (Eurobio), 2 mM L-glutamine (PAA) and antibiotics 100 μg/mL Penicilin, 100 μg/mL Streptomycin (Biovalley). The vesiculovirus spring viraemia of carp virus (SVCV) was produced at 20 °C on EPC cells in GMEM supplemented with 2% fetal bovine serum, 1% tryptose, 2 mM L-glutamine and antibiotics. The birnavirus Blotched snakehead virus (BSNV) was propagated as described in (Langevin et al., 2013b) at 20 °C on an *Ophiocephalus* cell line derived in the laboratory. BSNV does not replicate in EPC cells, while the reovirus Golden shiner virus (GSV) (Winton et al., 1987) can replicate, although with poor efficiency; the GSV was produced on another cell line from Fathead minnow, FHM cells (ATCC CCL42) at 24 °C.

2.7. Transfections

Twenty five millions of EPC cells were seeded on 6-well plates; the next day, cells were harvested from each well (about 4 millions cells per well), and were electroporated with 3–4 μg of plasmid per well using the nucleofector kit T (Lonza) following manufacturer's recommendations. Cells were kept for 3 days at 24 °C before proceeding with infections, poly I:C treatment or immunocytochemistry.

2.8. Virus infections

Virus absorption was performed on EPC cells in Glasgow's modified Eagle's medium-HEPES 25 mM medium (Eurobio) supplemented with 2% fetal bovine serum (FBS, Eurobio), 1% tryptose phosphate broth (Eurobio), 2 mM L-glutamine (PAA) and antibiotics 100 μg/mL Penicilin (Biovalley), 100 μg/mL Streptomycin (Biovalley) for 1 h at 14 °C. Four millions transfected EPC cells were seeded on a