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Neutralization of viral infectivity by zebrafish c-reactive protein isoforms

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ABSTRACT

This work explores the unexpected *in vivo* and *in vitro* anti-viral functions of the seven c-reactive protein (*crp1-7*) genes of zebrafish (*Danio rerio*). First results showed heterogeneous *crp1-7* transcript levels in healthy wild-type zebrafish tissues and organs and how those levels heterogeneously changed not only after bacterial but also after viral infections, including those in adaptive immunity-deficient *rag1*$^{-/-}$ mutants. As shown by microarray hybridization and proteomic techniques, *crp2*/CRP2 and *crp5*/CRP5 transcripts/proteins were among the most modulated during *in vivo* viral infection situations including the highest responses in the absence of adaptive immunity. In contrast *crp1*/CRP1/and *crp7*/CRP7 very often remained unmodulated. All evidences suggested that zebrafish *crp2-6*/CRP2-6 may have *in vivo* anti-viral activities in addition to their well known anti-bacterial and/or physiological functions in mammalians. Confirming those expectations, *in vitro* neutralization and *in vivo* protection against spring viremia carp virus (SVCV) infections were demonstrated by *crp2-6*/CRP2-6 using *crp1-7* transfected and/or CRP1-7-enriched supernatant-treated fish cells and *crp2-5*-injected one-cell stage embryo eggs, respectively. All these findings discovered a *crp1-7*/CRP1-7 primitive anti-viral functional diversity.These findings may help to study similar functions on the one-gene-coded human CRP, which is widely used as a clinical biomarker for bacterial infections, tissue inflammation and coronary heart diseases.

1. Introduction

Widely used as a general biomarker for bacterial infection and inflammation during many decades, circulating human pentameric CRP (pCRP) has been found recently within atherosclerotic lesions and might be used as a new biomarker for cardiovascular diseases (Shrivastava et al., 2015). Correlation between infections and cardiovascular heart diseases has been demonstrated not only for bacteria but also for several viral infections (Adinolfi et al., 2014; McKibben et al., 2016; Voulgaris and Sevastianos, 2016; Wu et al., 2016). Furthermore, although pCRP was initially discovered during acute-phase responses to bacterial infections increasing their circulating levels from < 10 to > 500 mg/l, intermediate concentrations of 10–50 mg/l were also detected during viral infections (Shah et al., 2015), suggesting that pCRP may have also anti-viral function(s). At this respect, viral infections induce human interferon alpha that represses the *crp* promoter, suggesting also pCRP antiviral effects (Enocsson et al., 2009). Nevertheless and despite pCRP being one of the most investigated risk biomarker molecule in the human cardiovascular field, and an important component of the anti-bacterial innate responses (Vilahur and Badimon, 2015), to our knowledge, there is no evidence yet that pCRP has any antiviral function.

In contrast to the one-gene *crp* of humans, zebrafish (*Danio rerio*) has 7 *crp* genes, from *crp1* to *crp7* (here simplified as *crp1-7* or CRP1-7 for their derived proteins). Amino acid variations among CRP1-7 proteins were mostly found in both their Ca^{++}-dependent phospholipid-binding pocket and conformational-domain sequences (Bello et al., 2017; Chen et al., 2015; Falco et al., 2012). By offering an easy-to-screen *in vivo* system for novel therapeutic molecules, zebrafish supplies a suitable model to explore CRP lipid-binding properties and conformation-dependent functionalities related to cardiovascular heart diseases including viral infections. Zebrafish is a well known model for heart development and function (Genge et al., 2016; Lu et al., 2016; Pitto et al., 2011) and a well known target for several fish rhabdoviruses (Encinas et al., 2013; Estepa and Coll, 2015a; Garcia-Valtanen et al., 2017; Varela et al., 2016). In this context, we have first explored *crp1-7*/CRP1-7 transcript/protein levels during several zebrafish viral infection situations and then designed several *in vitro*/*in vivo* strategies to explore

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crp1-7/CRP1-7 implication on viral infections.

Zebrafish CRP1-7 are made of protein monomers of ~ 200 amino acids (~23 kDa)(Chen et al., 2015; Falco et al., 2012). According to its proposed 3D structure, CRP5 is a trimeric Ca^{++}-dependent phospholipid-binding protein (tCRP) rather than a pentameric molecule (pCRP) as in humans (Chen et al., 2015). It is not yet known whether all the rest of zebrafish CRP isoforms are also trimeric (Bello et al., 2017) and/or whether they all have similar functionalities than human pCRP. For instance, although C1q (a known ligand of pCRP) have been identified in zebrafish (Boshra et al., 2006), fish have only IgM and one class of polymeric immunoglobulin receptor (PIGR)(Zhang et al., 2010) (other IgG receptors bind C1q-pCRP complexes). There have been no reports on interactions between CRP1-7 and zebrafish C1q or PIGR (Lu et al., 2012). Therefore, the possible human analogous functions of the CRP1-7 isoforms remain unknown.

Human and zebrafish CRPs showed a high degree of conservation, including the location of their two cysteine residues, and similarities between the amino acid sequences involved in their Ca^{++}-dependent ligand-binding pockets. Such conservation suggested similar functions in human pCRP and zebrafish tCRPs (Bello et al., 2017; Chen et al., 2015). On the other hand, the variations of amino acids around the ligand-binding pockets of zebrafish CRPs, suggested different ligand-binding specificities, which may be hypothetically explained by the need to target a wide pathogen diversity such as that found in aquatic environments (Bello et al., 2017). Previous preliminary data showing modulation of zebrafish *crp*-pathways during viral infections (Estepa and Coll, 2015a; Garcia-Valtanen et al., 2017) or trout *crp* upregulation during oral vaccination against virus (Ballesteros et al., 2012), suggested that zebrafish *crp1-7*/CRP1-7 may have some anti-viral activities. Because all the above mentioned reasons, we have further studied possible relations between zebrafish individual *crp1-7*/CRP1-7 and viral infections.

As zebrafish viral infection models we mainly chose two rhabdoviruses to which zebrafish is susceptible, the Spring Viremia Carp Virus (SVCV) (Lopez-Munoz et al., 2010; Sanders et al., 2003), and the Viral Haemorrhagic Septicemia Virus (VHSV) (Novoa et al., 2006). Rhabdoviruses penetrate into the fish body via their fins (Harmache et al., 2006). The progress of infection becomes externally associated with exophthalmia, abdominal distension, and petechial haemorrhages in fins and gills 3 to 6 days after penetration. A few days later, the most important fish internal lymphoid organs such as head kidney and spleen become also affected (Ahne et al., 2002; Ashraf et al., 2016). Mortalities are highest ~ 15 days after the beginning of infection (Encinas et al., 2013; Encinas et al., 2010).

To detect possible variations on *crp1-7*/CRP1-7 expression, we have explored several zebrafish infection situations. Thus, among the viral infection situations chosen, short-term (infection) and long-term responses (survival) were studied after infection with VHSV (Encinas et al., 2010; Estepa and Coll, 2015b) and SVCV (Encinas et al., 2013). Bacterial infections were also studied because of the well known antibacterial pCRP responses on humans (Kindmark, 1971). Since resistance to viral infections in both fish and mammalians depends both on innate and adaptive responses (i.e., neutralizing IgM antibodies in fish and both IgM/IgG antibodies in mammalians), fish rely more heavily in innate than in adaptive responses to fight viral infections (Sunyer, 2013; Sunyer et al., 1998). To explore the importance of *crp1-7* innate responses in the presence and absence of adaptive immunity, we studied adaptive immunity-deficient zebrafish *rag1*$^{-/-}$ mutants, which have no antibodies nor T-cell receptors and whose responses to viral infections have been studied recently (Garcia-Valtanen et al., 2017). Results showed heterogeneous *crp1-7* transcriptional profiles in all the above mentioned infection situations including higher responses in the absence of adaptive immunity, all results suggesting heterogeneous *crp1-7* anti-viral responses. Confirming those expectations, *in vitro* neutralization and *in vivo* protection of SVCV infection were found for the first time to be induced by the different zebrafish *crp1-7*/CRP1-7 isoforms. In addition to its possible implications to prevent and/or to treat human cardiovascular/viral diseases, this knowledge and future studies on their mechanism(s) of action may help to understand primitive vertebrate CRP diversity and how it may have evolved to humans. It also could be applied to improve prevention methods for viral infection in farmed fish.

2. Material and methods

2.1. Zebrafish (Danio rerio)

Adult XL wild type zebrafish of 700–900 mg of body weight (3–4 cm in length) were obtained from a local pet shop (Aquarium Madrid, Madrid, Spain). Zebrafish of 6 months of age (~ 500 mg of body weight) with truncated-inactivated recombinant activation gene (*rag1*$^{-/-}$) and their corresponding wild-type *rag1*$^{+/+}$ counterparts were originally obtained from David Raible's fish facility at the University of Washington (USA) and raised, maintained, and characterized as described before (Garcia-Valtanen et al., 2017). Zebrafish were maintained at 24–28 °C in 30 l aquaria with tap-dechlorinated carbon-filtered water with 1 g of $CaCl_2$, 1 g of $NaHCO_3$ and 0.5 g of Instant Ocean sea salts added to water resulting in a conductivity of 200–300 μS and pH of 7.8-8.2. The aquaria were provided with biological filters and fish fed daily with a commercial feed diet (Vipan Bio-Vip, Sera, Heisenberg, Germany). Previously to the viral infection challenge, fish were acclimatized for 2 weeks to the corresponding optimal viral replication temperatures.

2.2. Fish cell culture

The *epithelioma papulosum cyprinid* (EPC) cells from the fathead minnow fish (*Pimephales promelas*) were obtained from the American Type Culture Collection (ATCC, Manassas, Vi, USA, code number CRL-2872). EPC cell monolayers were grown at 28 °C in a 5% CO_2 atmosphere in RPMI-1640 Dutch modified culture medium (Gibco, UK) supplemented with 20 mM HEPES, 10% fetal bovine serum, FBS (Sigma, St. Louis, USA), 1 mM piruvate, 2 mM glutamine, 50 μg/ml of gentamicin (Gibco) and 2 μg/ml of fungizone.

2.3. In vitro infections with viral haemorrhagic septicemia virus (VHSV) and spring viremia carp virus (SVCV)

The fish *novirhabdovirus* viral haemorrhagic septicemia virus (VHSV) strain 07.71 (accession number AJ233396) isolated from rainbow trout *Oncorhynchus mykiss* (LeBerre et al., 1977) and the rhabdovirus Spring Viremia Carp Virus (SVCV) isolate 56/70 from carp *Cyprinus carpio* (Fijan et al., 1971), recently renamed *Carp Sprivivirus* (ICTV, 2015), were used for *in vitro* and *in vivo* infections. VHSV or SVCV were replicated in EPC cell monolayers at 14 °C (Estepa and Coll, 2015a) or at 22 °C (Garcia-Valtanen et al., 2017), respectively, in the cell culture media described above except for 2% FBS (infection media) and absence of the CO_2 atmosphere. Supernatants from VHSV or SVCV-infected EPC cell monolayers were clarified by centrifugation at 4000 g for 30 min and kept at −80 °C. *In vitro* viral infections were performed by 2 h adsorption of the viral supernatants to the EPC cell monolayers, followed by washing the unbound viruses with infection media and incubation at their respective optimal replication temperatures during 24 h. The infected EPC cell monolayers were fixed and viral titers assayed by the *in vitro* by the focus forming units (ffu) assay as described before (Chinchilla et al., 2013b).

2.4. In vivo infections of adult zebrafish with VHSV, SVCV and bacteria

The procedures used for infecting zebrafish with VHSV or SVCV viruses were described before. Briefly, zebrafish were acclimatized to 14 °C for VHSV infection or to 22 °C for SVCV infection during 2 weeks