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Structure and functionalities of the human c-reactive protein compared to the zebrafish multigene family of c-reactive-like proteins

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ABSTRACT

Because of the recent discovery of multiple c-reactive protein (*crp*)-like genes in zebrafish (*Danio rerio*) with predicted heterogeneous phospholipid-binding amino acid sequences and heterogeneous transcript expression levels in viral survivors and adaptive-deficient mutants, zebrafish constitute an attractive new model for exploring the evolution of these protein's functions, including their possible participation in fish trained immunity. Circulating human CRP belongs to the short pentraxin family of oligomeric proteins that are characteristic of early acute-phase innate responses and is widely used as a clinical inflammation marker. In contrast to pentameric human CRP (pCRP), zebrafish CRPs are trimeric (tCRP); however monomeric CRP (mCRP) conformations may also be generated when associated with cellular membranes as occurs in humans. Compared to human CRP, zebrafish CRP-like proteins show homologous amino acid sequence stretches that are consistent with, although not yet demonstrated, cysteine-dependent redox switches, calcium-binding spots, phosphocholine-binding pockets, C1q-binding domains, regions interacting with immunoglobulin Fc receptors (FcR), unique mCRP epitopes, mCRP binding peptides to cholesterol-enriched rafts, protease target sites, and/or binding sites to monocyte, macrophage, neutrophils, platelets and/or endothelial cells. Amino acid variations among the zebrafish CRP-like multiprotein family and derived isoforms in these stretches suggest that functional heterogeneity best fits the wide variety of aquatic pathogens. As occurs in humans, phospholipid-tagged tCRP-like multiproteins might also influence local inflammation and induce innate immune responses; however, in addition, different zebrafish tCRP-like proteins and/or isoforms might fine tune new still unknown functions. The information reviewed here could be of value for future studies not only to comparative but also medical immunologists and/or fisheries sectors. This review also introduces some novel speculations for future studies.

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1. The human CRP short-pentraxin family

Human c-reactive protein (CRP) belongs to a family of short-chain pentraxins that share > 50% amino acid identity and some structural/functional features with the serum amyloid P component (SAP) (Woo et al., 1985). Blood circulating human CRP shows a pentameric (pCRP) cyclic symmetry which is formed by monomers of ~200 amino acids (~23 KDa) (Shrive et al., 1996). Other minority conformations such as mild-acidic-pH pentamers (Hammond et al., 2010) or decamers and tissue-associated monomers (mCRP), have also been described (Wu et al., 2015).

CRP is a part of the innate response and is released mainly from the liver into the blood, triggered by any cellular damage produced by an injury and/or disease to affect systemic/local inflammation and help in pathogen neutralization (Zhang et al., 2010). CRP as a part of the acute-phase response increases its basal plasma level 2–3 orders of magnitude (i.e., from 1 to 5 to 500–1000 μg per ml) hours after the inflammatory signals are generated (Bottazzi et al., 2016; Du Clos, 2013; Vilahur and Badimon, 2015). Therefore it has been widely used as a biomarker for inflammation (Pepys et al., 2006).

Human pCRP is arranged in flat pentamers with one face showing Ca^{++}-dependent phospholipid-head recognition and the other face being responsible for most of their biological effects. After binding ~1 mM Ca^{++}, pCRP recognizes membrane exposed phospholipid-head ligands primarily containing phosphorylcholine heads with μM affinities, while pSAP preferentially binds phosphorylethanolamine heads (Du Clos and Mold, 2011). The Ca^{++}-dependent binding of pCRP to phospholipid heads, triggers binding to C1q (activating the classical complement pathway) and immunoglobulin Fc receptors (FcR) (activating phagocytosis) (Bang et al., 2005; Du Clos and Mold, 2011; Inforzato et al., 2013; Vilahur and Badimon, 2015). After the discovery of different monomeric CRP (mCRP) conformations, the list of ligands has expanded (Li et al., 2016; Wu et al., 2015), as conformational changes control not only local inflammation but also many different innate immune responses (Wang et al., 2011; Wu et al., 2015) (further details are discussed later in section 5).

CRP-like proteins are found from arthropods to mammalians (Bottazzi et al., 2016; Vilahur and Badimon, 2015), including lower vertebrates such as teleost fish (MacCarthy et al., 2008; Pionnier et al., 2013, 2014). However, CRP proteins have been mostly studied in humans because of their clinical implications. In this work, we will compare the unique properties of the human *crp* gene/CRP protein with those of the zebrafish *crp*/CRP multi-gene/multi-protein family with respect to their sequences, protein structure and biological functionalities.

2. The CRP-like multi-gene family in zebrafish

One of the zebrafish CRP-like recombinant protein members (similar to CRP5) has a similar backbone structure to human CRP but crystallizes as a trimer with only 32% amino acid identity (Chen et al., 2015). In sharp contrast to human and other fish CRP-like proteins, 7 different *crp* loci (coding for CRP1-7) were detected in chromosome 24 of the zebrafish (*Danio rerio*) genome (Falco et al., 2012). Furthermore, zebrafish *crp5* expresses 79 different transcript variants suggesting the existence of at least 5 different isoforms of this particular gene (GenBank UniGene accession numbers Dr.124528-Dr.162306) (Chen et al., 2015). In addition, mRNA transcripts identified as 4 *sap*-like genes in earlier home-designed microarrays (Encinas et al., 2010, 2013; Estepa and Coll, 2015) have now been confirmed to be isoforms of *crp2* and *crp5* in most recent genome releases (not shown). Caution is necessary when assigning *crp/sap* identities based only on their sequences, especially when searching for orthologous sequences from other species such as human CRP, because of their high similarity. Classification is best based on affinity or ligand-binding functionality rather than sequence (i.e., human CRP preferentially binds phosphocholine while SAP binds phosphoethanolamine, and other molecules could have other preferences) (Du Clos and Mold, 2011). Although it is likely that there might be more *crp*-like genes and/or isoforms in zebrafish, carp and/or other fish (Fujiki et al., 2001), their complete identification must wait for further sequencing advances of their corresponding genomes and/or transcripts. The existence of a *crp*-like multi-gene family rather than a unique gene could offer an alternative repertoire of anti-pathogen responses for primitive vertebrates such as fish that have a limited adaptive immune response (i.e., lack of IgG). However, the corresponding zebrafish CRP-like multi-protein function(s) and regulation(s) remain largely unexplored.

Zebrafish is a suitable model for studying evolutionary immunology since it has been used not only for modelling other vertebrates due to its important resources for genetic and developmental studies but also for modelling many infectious diseases affecting fish (Chinchilla et al., 2015; Lopez-Munoz et al., 2010; Novoa et al., 2006; Rowe et al., 2014; Sanders et al., 2003) and humans (Goody et al., 2014). In addition, transcriptomic data showing differentially modulated *crp1-7* multi-gene has been reported in zebrafish surviving rhabdoviral infections such as VHSV (Estepa and Coll, 2015), or SVCV (Encinas et al., 2013), bacterial infections (Estepa and Coll, 2015) and/or adaptive-immune deficient zebrafish (Garcia-Valtanen et al., 2016). Some of these results will be discussed in more detail in section 6.

3. Comparative amino acid sequences of human CRP and zebrafish CRP-like proteins

A sequence alignment of the known CRP proteins from various species revealed CRP-like protein sequences from fish clustered together in a phylogenetic tree, while those from mammalians formed a distinct clade and *Limulus* was out grouped (Falco et al., 2012). Both ^{36}Cys and ^{97}Cys and the cholesterol-binding sequence (residues 35 to 47) were well conserved throughout evolution

(Table 1), suggesting that at least some of their functions (to be discussed in section 5), such as redox switching or interactions with cholesterol-enriched rafts, might be similar (Chen et al., 2015; Wang et al., 2011). A high degree of conservation was also observed when comparing the protein hydrophobicity profiles between human CRP and zebrafish CRP-like proteins (see Fig. 1A for CRP5 and not shown). In addition to the two cysteine residues, the locations of Ca^{++}-binding and putative phospholipid-binding pockets were highly conserved (Fig. 1B, Table 1). Variation of amino acid residues among zebrafish CRP1-7 showed a conserved 26–39 amino acid stretch around ^{36}Cys suggesting a common structural/functional requirement (possibly a cholesterol-binding sequence). The highest variations were found around the putative phospholipid-binding pocket (residues 70–86) and the C-terminal region (residues 180–206), suggesting different possible specificities for ligand binding (Fig. 1B, Table 1).

4. Tridimensional structures of human CRP and zebrafish CRP-like proteins

The X-ray derived structures of crystalized human CRP are pentameric (Shrive et al., 1996). Circular dichroism, infrared spectroscopy and/or immunochemistry suggest Ca^{++}-dependent reversible small conformational changes (Ramadan et al., 2002; Wu et al., 2015). Furthermore, in the presence of Ca^{++} the pCRP structures become resistant to heat or urea denaturation, mercaptoethanol and/or proteolysis (Black et al., 2003; Coll, 1988). In sharp contrast, the X-ray structures of zebrafish CRP-like proteins were trimeric (tCRP), with a small central pore of ~16.8 Å compared to human pCRP, which is ~47 Å. Small Ca^{++}-dependent conformational changes were also shown for one of the zebrafish tCRP-like proteins (Chen et al., 2015) but no Ca++-dependent or any other cation-dependent binding specificities have been reported to date.

Each human CRP monomer binds 2 molecules of Ca^{++} at a binding site for the negatively charged phosphate moiety of phospholipid ligand heads. The loss of Ca^{++} results in a highly charged negative surface of the Ca^{++}-binding loop (Fig. 1A, green squares) activating binding to ligands such as polycations (i.e., polyLys). The major significant change between Ca^{++}-depleted and Ca^{++}-bound human CRP occurs in the Ca^{++}-binding loop (amino acids 138–150) (Fig. 1A, blue squares), which are disordered and mobile in the absence of Ca^{++}, and refold in the presence of Ca^{++} to protect the ^{145}Asn-^{147}Glu site (Fig. 1A red triangles) from proteolytic attack (Kinoshita et al., 1992).

The location of the highest amino acid sequence variations (Fig. 1B, Table 1) and homology docking-modelling (Chen et al., 2015) of the phospholipid pockets of different zebrafish CRP-like proteins, suggest that different specificities for phospholipid head binding might exist for each of them. However, to date there is no experimental evidence that could confirm these indirect observations.

Accumulating evidence, however, confirms that human CRP has two forms, pentameric serum-circulating CRP (pCRP) and monomeric tissue-associated CRP (mCRP) (Wu et al., 2015). The mCRP might be induced *in vivo* by interactions between pCRP and cholesterol-enriched lipid rafts in the membranes of damaged cells (Wang et al., 2011) (further discussed in section 5) or direct biosynthesis from macrophages (Ciubotaru et al., 2005). *In vitro* the pCRP to mCRP has been induced in the absence of Ca^{++} (presence of EDTA) with urea, low-pH or low-salt buffers (Potempa et al., 1983; Taylor and van den Berg, 2007).

The conversion of human pCRP to mCRP is required for an additional conformational change, due to reduction of the intra-monomer ^{36}Cys-^{97}Cys disulfide bond as detected by its slower migration in SDS-denaturing gel electrophoresis and/or specific monoclonal antibodies (Wang et al., 2011). Reducing agents such as DTT, GSH, Cys or mercaptoethanol can reduce mCRP but not pCRP, since the disulfide bond is protected inside the pentameric structure (Shrive et al., 1996). Once reduced, spontaneous re-oxidation of mCRP that could complicate the interpretation of the results, can be prevented by treatment with *N*-ethylmaleimide or, alternatively, by substituting the two Cys for Ala in recombinant CRP (Ji et al., 2007; Potempa et al., 2015). The reduction affects some of the human mCRP biological functions (Wang et al., 2011; Wu et al., 2015), but no X-ray structures have been yet reported for non-reduced or reduced conformations to date.

Therefore, the participation of every possible conformation of CRP (pCRP, mCRP and/or reduced mCRP) should be correctly

Table 1
Some reported activities of amino acid sequences and/or peptides from human and zebrafish CRP.

Activity	Human amino acid sequence Zebrafish amino acid sequence
Redox switch	^{36}C + ^{97}C ^{36}C + ^{98}C
pCRP Ca^{++} main contacts	^{60}**D**N^{61} + ^{138}EQ**D**^{140} + ^{147}EGS**Q**^{150} ^{60}**D**E^{61} + ^{139}DP**D**^{141} + ^{148}DVD**Q**^{151}
pCRP phosphocholine binding peptide & sites	^{47}**R**GYSI**FSY**A**T**KRQ**D**NEI^{63} + ^{68}S + ^{76}T + ^{138}E + ^{140}**D** + ^{149}**S** ^{47}**R**EVIL**FAYYT**PDV**D**ELN^{63} + ^{67}E + ^{75}Y + ^{139}D + ^{141}**D** + ^{152}**S**
C1q binding	^{38}H + ^{88}E + ^{112}**DGK**^{114} + ^{158}**N** + ^{175}**YL**^{176} ^{38}R + ^{89}P + ^{113}**DGR**^{115} + ^{159}**N** + ^{176}**YY**^{177}
mCRP binding to Ch, C1q, LDL, fibronectin,collagen	^{35}V**C**LHFY**TEL**SST**R**^{47} ^{35}L**C**MRVA**TEL**PLD**R**^{47}
Implicated in mCRP-mCRP interactions	^{40}Y**TEL**SS^{45} + ^{91}VAPV^{94} + ^{109}**FW**V**DG**KPRVRK^{119} + ^{167}**S**PDE**I**NTI**Y**^{175} ^{40}A**TEL**PL^{45} + ^{91}STLQ^{94} + ^{110}**FW**M**DG**RRSLHQ^{120} + ^{168}SSAQ**I**KAV**Y**^{176}
Unique mCRP epitope	^{201}**G**E**V**FTK**P**^{207} ^{205}**GN**V**L**V**V**P^{211}
Protease sensitive sequence	^{145}N**F**E^{147} ^{146}SFD^{148}
Highest amino acid variations on zebrafish CRP-like sequences	^{70}**D**I**G**Y**S**FTVGG–SEIL**F**EV^{86} ^{69}DGRV**S**LYIQSSKDAAF**F**RL^{87}
Lowest amino acid variations on zebrafish CRP-like sequences	^{18}**SYV**S**L**KAPLTKP**LKAFTV**CLHF^{39} ^{18}**SYV**K**L**YPEKPLS**L**S**AFTL**CMRV^{39}

Human CRP sequence 1GNH.pdb and zebrafish CRP5 sequence from Protein Bank accession number 4PBP.pdb (GenBank accession number JF772178.1) were aligned and numerated without the signal peptides as reported before (Chen et al., 2015; Shrive et al., 1996). Compiled from several published data (Chen et al., 2015; Li et al., 2016; Shrive et al., 1996; Wang et al., 2011; Wu et al., 2015; Ying et al., 1989) and this work. **Bold**, identical amino acids between human CRP and zebrafish CRP5. **Underlined**, location of amino acid variations among different zebrafish CRP-like 1–7 molecules.

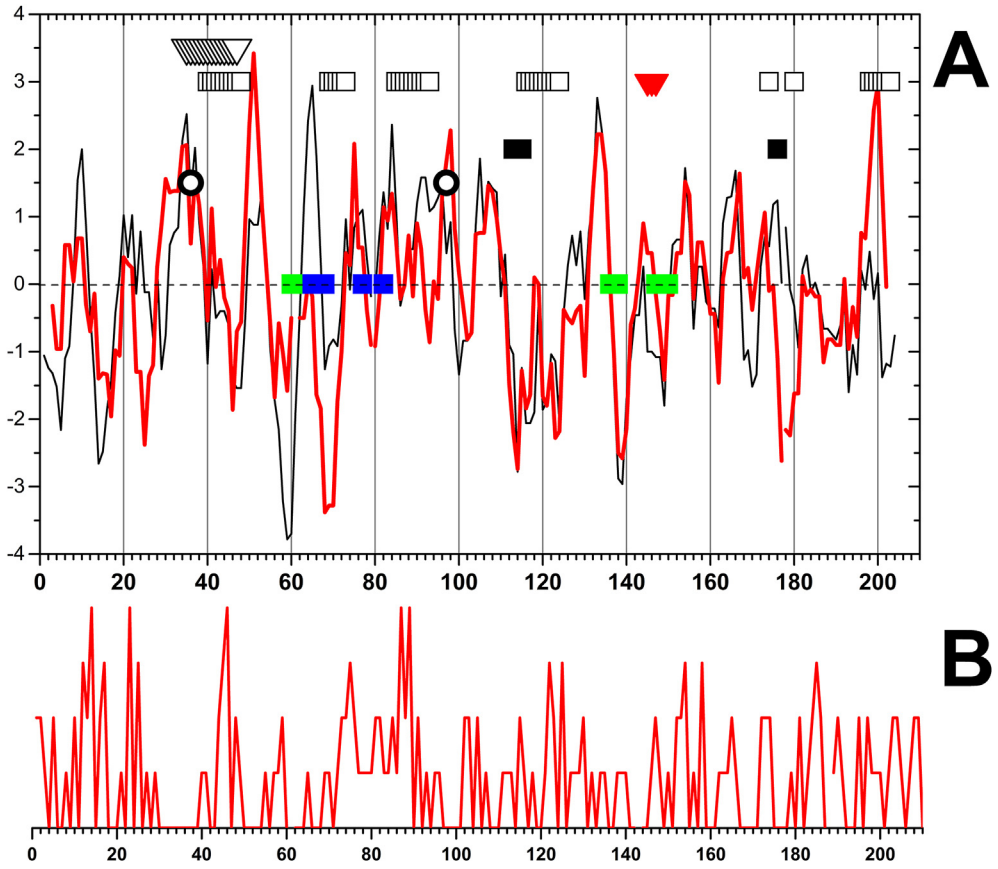

Fig. 1. Hydrophobicity map (A) and variability (B) of human and zebrafish CRP amino acid sequences. A) The hydrophobicity map of human and zebrafish CRP (accession numbers 1GNH and 4PBP from the RCSB PDB Protein Data Bank at http://www.rcsb.org/pdb/home/home.do, respectively) were derived using Clone Manager vs9 software. The crystalized zebrafish CRP 4PBP (GenBank accession number JF772178.1) (Chen et al., 2011) is highly similar to CRP5 (Falco et al., 2012). The numbering correspond to the amino acid sequences without their signal peptide. **Black line**, human CRP. **Red line**, zebrafish CRP. **Black circles**. location of cysteines (^{36}Cys and ^{97}Cys). **Green squares**, amino acids involved in human pCRP-Ca^{++} binding. **Open squares**, amino acids involved in the intermolecular monomer (mCRP) to mCRP interactions to form pCRP. **Red triangles**. protease sensitive sequence. **Blue squares**, amino acids involved in ligand (PC)-CRP-binding. **Blue circles**, predominant epitope (197–202) expressed only in mCRP but imbedded in the inter-monomer regions in pCRP (Wu et al., 2015). **Black squares**, amino acids involved in C1q-binding (Chen et al., 2015; Shrive et al., 1996). **Open triangles**, sequence implicated in the binding to cholesterol-enriched lipid rafts (residues 35–47) mediating mCRP insertion and signaling (Wu et al., 2015). **B)** Number of different amino acids per position in zebrafish CRP1-7 gene derived proteins (accesion numbers XM_693995, BC097160, BC154042, BC115188, BC121777, BC162745, BC150371, respectively) (Falco et al., 2012). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

determined before drawing any valid experimental conclusions. In addition, the fact that most commercially available anti-CRP antibodies recognize both pCRP and mCRP (Schwedler et al., 2006), complicates the interpretation of previous and/or future studies. Similar concerns will apply to research on zebrafish CRP-like proteins although there is no evidence that the tCRP to mCRP conversion also occurs in any zebrafish CRPs.

5. Biological functions of CRP

The presence and some functions of CRP-like (Bayne and Gerwick, 2001; Bayne et al., 2001) and SAP-like (Hwang et al., 2015; Wang and Sun, 2016) proteins have been described in several fish but not in the zebrafish CRP multi-protein family. Several biological functions which might be expected to be found in the zebrafish CRP multi-proteins will be briefly discussed here.

5.1. Inflammation biomarker of CRP circulating levels

Human local tissue injuries cause (even hours later) the release of pro-inflammatory signals such as IL6, IL1β and TNFα, which distribute throughout the body and induce the liver to synthesize and release pCRP to the blood. The participation of these earliest signals has raised some possibilities for the clinical control of pCRP blood levels to reduce excessive tissue damage using antibodies directed against pro-inflammatory signaling molecules. Three examples of such antibodies are Tocilizumab (anti-human IL6R), Rilonacept (anti-human IL1R), and Certolizumab (anti-TNF-alpha). In contrast, similar fish studies are very scarce (Bayne and Gerwick, 2001; Bayne et al., 2001). Nevertheless, the analysis of differential expression data of transcription factors present in previous zebrafish microarray studies (Estepa and Coll, 2015), might help to identify some of the regulation of the *crp1-7* promoters in zebrafish surviving viral diseases.

5.2. Ca^{++}-dependent recognition of exposed phospholipid heads

The similar relative locations and partial amino acid identities between the amino acid stretches in human and zebrafish CRP involved binding Ca^{++} (residues 60–61, 138–140, and 147–150 in human CRP) and phosphocholine (17-mer peptide 47–63 and residues 68, 76, 138, 140, and 150 in human CRP) (Table 1) strongly suggested that similar functionalities exist in both human and zebrafish monomers. However, Ca^{++}-dependent binding to exposed membrane phosphocholine *in silico* modelled with different zebrafish CRP-like monomers identified different binding

pocket geometries for the different CRPs (Chen et al., 2015). Preliminary extension of similar *in silico* binding energy data to different phospholipid heads has also shown heterogeneity among the different zebrafish CRP-like proteins (not shown) and requires further refinement studies. Validation of such *in silico* data can be performed by ELISA using solid-phases coated with different phospholipid-heads (Martinez and Coll, 1987, 1988) or CRP pepscans (Garcia-Valtanen et al., 2014) aas presently being attempted (results not shown). In addition, interactions of zebrafish CRP-like conformations (tCRP, mCRP, reduced-mCRP) could not only be studied by ELISA/pepscan methods but also with fish cells and/or model membranes. Infected fish cells might offer a new exciting exploration subject for PCR-like bindings because VHSV infection induced translocation/exposure of cellular phosphatidylserine heads (Estepa et al., 2001).

5.3. Binding to C1q and complement components

Once human pCRP binds phospholipid-head exposed targets, it binds C1q to activate the classical complement pathway (Agrawal et al., 2001; Agrawal and Volanakis, 1994; Gaboriaud et al., 2003; Thompson et al., 1999). The binding of human CRP to apoptotic cells in damaged tissues in a Ca^{++}-dependent manner affects not only complement but also innate immune responses. Thus, CRP-"marking" of apoptotic cells increased the classical pathway of complement activation but protected the cells from assembly of the terminal complement components avoiding excessive tissue damage. Furthermore, apoptotic cells "marked" with CRP enhanced their opsonisation and phagocytosis by anti-inflammatory TGFβ-activated macrophages. The anti-inflammatory effects of CRP required C1q and factor H. However, reduction of the disulfide bridge enhanced the interaction of mCRP with C1q (Wu et al., 2015), a novel interaction which is much less studied. All the above observations demonstrate that CRP and some complement components act in concert to promote non-inflammatory clearance of apoptotic cells (Gershov et al., 2000). The similar relative locations and partial amino acid identities in 5 different stretches involved in human CRP binding to C1q (residues 38, 88, 112–114, 158, 175–176) strongly suggest similar functionalities might exist in pCRP and tCRP (Table 1). C1q and other complement components have been identified in zebrafish (Boshra et al., 2006), and most recently the crystal structure of one of the globular domains of zebrafish C1q which might be implicated in CRP-binding was elucidated (Yuan et al., 2016). However, how the different tCRP-like proteins from zebrafish might recognize and interact with C1q could only be modelled until more experimental data will be available.

5.4. Binding to immunoglobulin Fc

Once human CRP binds phospholipid-head exposed targets, binds to Fc transmembrane immunoglobulin receptors (FcR) present in most human haematopoietic cells, enhancing FcR^{+} leukocyte phagocytosis of bacteria (Kindmark, 1971). Each pCRP bound one specific FcR molecule (Lu et al., 2012), two monomers participating in the binding (Lu et al., 2008) (PDB entries 1F2Q and 1QVZ for FcεRI and FcαRI, respectively). For instance, human pCRP binds to FcɣRIIa (*cd32*) and FcɣRI (*cd64*) while mCRP binds to FcRIIIb (Wu et al., 2015). Internalization of CRP-FcR caused immunosuppression in macrophages, suggesting that CRP/FcR complexes might also protect against excessive inflammation (Marjon et al., 2009). However, none of those described human Fc receptors were IgM-specific, while IgM is the primary fish immunoglobulin. In addition, only polymeric immunoglobulin (*pig*) receptor (*pigr*) genes have been identified in zebrafish (Zhang et al., 2010). Transcript levels of *pig* genes were down-regulated after infection with Snakehead rhabdovirus, suggesting that viral infection may suppress them (Kortum et al., 2014), but there are few other reports on interactions of fish tCRP-like proteins with FcRs (Bayne and Gerwick, 2001; Bayne et al., 2001; Lu et al., 2012) and none with mCRP conformations (Li et al., 2016). In this context, it will be of interest to study CRP-like/FcR interactions in zebrafish *rag1*$^{-/-}$ mutants lacking IgM and expression of transcripts of *pig/pigr* in microarray studies.

5.5. Changing conformations after binding to cholesterol-enriched lipid rafts

The most important human CRP peptide implicated in cholesterol-enriched lipid raft binding (residues 35–47) is found in a similar relative location with 38.4% partial amino acid identity in zebrafish CRP-like molecules (Table 1 shows correspondences between human and zebrafish CRP5). Similarly, the main stretches participating in human mCRP interactions to form pCRP could also be found in CRP5 (Table 1, residues 40–45, 91–94, 109–119, 167–175 in human CRP). Both findings suggest that human and zebrafish CRPs may use similar mechanisms for CRP cholesterol-induced conformations. The Ca^{++}-dependent binding of human CRP to damaged cell membranes enhances the pCRP dissociation to mCRP (Ji et al., 2007). The pCRP dissociation could be due to interactions with hydrophobic cholesterol-enriched lipid rafts as shown by mimicking with model membranes made with cholesterol, phosphatidylcholine and sphingomyelin (Potempa et al., 2015; Wang et al., 2011). Furthermore, evidence is accumulating that the changes in the pCRP conformation further alter its biological effects (Wu et al., 2015). In human CRP, this generates an expanded list of not only those well-known interactions such as C1q/Fc but also with new ligands such as LDL, fibronectin, collagen (Li et al., 2016) and/or many other possibilities which have not yet been identified.

5.6. In vivo reduction of the cystein switch

The relative locations of the two Cys residues present in each of the CRP monomers were highly conserved in human and all zebrafish CRP-like molecules (residues 36 and 97 or 36 and 98). After losing the pentameric symmetry, the human mCRP intramonomer disulfide bridge acts as a redox-sensitive switch whose reduction might be required for the expression of further pro-inflammatory effects (Wang et al., 2011). *In vivo* interactions of pCRP with cholesterol-enriched lipid rafts not only activates the conversion to mCRP but also the reduction of the disulphide bond (Li et al., 2016; Wang et al., 2011). Since human mCRP promotes recruitment of thioredoxin (Trx)-rich monocytes/macrophages (Eisenhardt et al., 2009a), Trx might be responsible for the ~1000-fold enhancement of the disulfide reduction rate compared to chemical reagents (Wang et al., 2011). In this respect, immunohistochemical studies with specific MAbs detected mCRP rather than pCRP in human atherosclerotic plaques (Eisenhardt et al., 2009a, 2009b, 2009c). Reduced mCRP has also been associated with enhanced binding to LDL (Ji et al., 2006b), further activation of complement (Ji et al., 2006a; Wang et al., 2011) and stimulation of innate immune responses of endothelial cells (Heuertz et al., 2005; Ji et al., 2009). While there are no similar confirmatory studies made yet on this recently described phenomenon on any zebrafish tCRP-like molecules, it is probable that their Cys behave in similarly despite being in a trimer.

5.7. Cellular interactions

Little is known about how the changes in the recently described human pCRP conformations may affect cellular responses. Furthermore, while there are many studies on the effects of human CRP on different cells and cellular lines, the results are sometimes contradictory and in any case difficult to interpret. Very often, the CRP preparations used for the experimentation could not be completely characterized (i.e., composition of pCRP, mCRP, reduced-mCRP) at the time the experiments were made. Nevertheless, recent evidence suggests that the effects of human mCRP are mostly pro-inflammatory, through the interaction with monocyte/macrophages, neutrophils, platelets, and/or endothelial cells (Khreiss et al., 2004, 2005; Wu et al., 2015). No studies have been yet made with any zebrafish tCRP-like molecules but if the corresponding recombinant proteins can be purified and their preparations characterized, protease digestion (Coll, 1988), pepscan mapping (Chico et al., 2010; Li et al., 2016) and specific cellular assays (Garcia-Valtanen et al., 2014) could be then applied to map possible heterogeneous cellular functionalities.

6. Do zebrafish CRP-like genes belong to some of the molecules recently implicated in “trained immunity”?

Recent evidence reveals that vertebrate innate immunity has a stronger and more rapid response to a second pathogen exposure, two characteristic properties of classical adaptive immune memory responses (Netea et al., 2016), constituting the so-called trained immunity (Netea et al., 2011). Because of their primitive immunological system among vertebrates (i.e., immunological memories showing only shorter lag-time rather than higher level responses, no IgG switch, no IgM maturation, mucosal IgT, phagocytic B-cells, etc.), fish offers a suitable model in which to study immune evolution (Bengten and Wilson, 2015; Sunyer, 2013). A first observation suggesting different functions for the fish *crp*-like genes and/or isoforms, was their tissue expression heterogeneity in carp (Falco et al., 2012). Confirming this possibility, transcriptional microarray and RTqPCR profiles of zebrafish lymphoid organs, showed that *crp1-6-* transcripts (except *crp7*), were down-regulated in survivors to viral haemorrhagic septicemia virus (VHSV) before and after VHSV re-infection, while they remain mostly un-modulated shortly after the first VHSV infection (except *crp7*) (Fig. 2). Furthermore, only *crp2, crp5* and *crp6* transcript levels increased when survivors were re-infected (compare the corresponding open and hatched yellow bars with * in Fig. 2). Down-regulation of most *crp*-like genes in VHSV survivors before and after re-infection was a surprising finding that might reflect a rapid cell migration to the viral entry sites. Nevertheless, these observations were the first description of *crp*-like transcript bacterial responses in viral-infected fish (Estepa and Coll, 2015). The expression heterogeneity of *crp*-like genes was confirmed in zebrafish *rag1*$^{-/-}$ mutants defective in V(D)J recombination in IgM and T-cell receptor genes (Garcia-Valtanen et al., 2016). Transcript upregulation of a unique trout *crp*-like gene was also induced by oral DNA-vaccination to virus (Ballesteros et al., 2012), confirming the importance of CRP-like proteins in fish immunization to viruses. Because, some of the mammalian orthologous to fish multi-gene families (i.e., *nitr*) were linked to long-term NK-cells in mammalian trained immune memory (Martin-Fontecha et al., 2004), the analogous *crp*-like family might also be responsible for similar purposes. As further

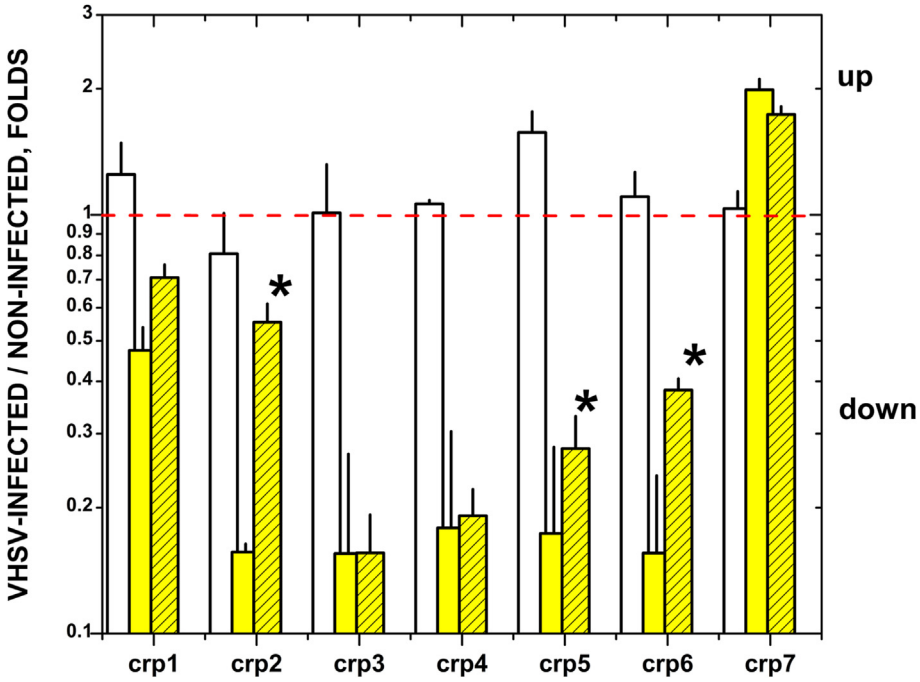

Fig. 2. Example of heterogenous differential expression profiles of individual *crp* gene transcripts from zebrafish surviving VHSV infections. Adult zebrafish were infected by VHSV and some of them analysed 2 days later. Survivors were maintained during 6 months and analysed before and after being re-infected with VHSV 2 days later. For the analysis, zebrafish lymphoid organs (head kidney + spleens) were harvested, their RNA extracted, fluorescently labelled and hybridized to zebrafish *crp1-7* probes in a home-designed microarray. The transcript differential expression was calculated for each *crp* gene using the following formula: fluorescence of each VHSV-infected replicate/mean fluorescences from non-infected replicates (n = 4 zebrafish pools of 5 fish per pool). Means and standard deviations were then obtained for each gene to remove outliers and calculate final folds. **Open bars**, 2-days after VHSV infection. **Yellow open bars**, 6 month survivors after 2 consecutive VHSV infections. **Yellow hatched bars**, 6 month survivors, 2 days after being re-infected. **Red horizontal hatched line**, 1- fold thresholds. **Folds>1**, up-regulated. **Folds<1**, down-regulated *, significantly different from non-re-infected VHSV survivors (yellow open bars) at the $p < 0.05$ level (Student T). Re-drawn from previously published data using unique 60-mer probes from zebrafish CRP1-7 mRNA (accesion numbers XM_693995, BC097160, BC154042, BC115188, BC121777, BC162745, BC150371, respectively) (Estepa and Coll, 2015). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

discussed in previously published (Estepa and Coll, 2015) and submitted work (Garcia-Valtanen et al., 2016), because of its heterologous expression within microarray data and the existence of multiple genes and/or transcript isoforms similar to in the *nitr* family, zebrafish tCRP-like molecules might be one of the novel actors of trained immunity in fish (see Fig. 2).

7. Conclusions

None of the novel aspects of the functionality of human mCRP molecules have been studied in zebrafish. It is unknown whether zebrafish tCRP-like molecules behave as serum acute-phase proteins, although this has been observed in other fish (Bayne and Gerwick, 2001; Bayne et al., 2001) and preliminary experiments using viral-infected zebrafish (unpublished). In addition to the different binding specificities of the different CRP1-7 and its transcript variants, because of the possible co-existence of different CRPs in the same cell, the generation of heterologous tCRP may further increase its potential functionalities. The sequence diversity of the *crp*-like genes combined with their possible different phospholipid-binding affinities and the possibility of heterologous tCRP, suggests this gene family could mimic a limited antibody repertoire. The heterogeneous transcript expression demonstrated after a viral infection, in viral survivors and in the absence of any adaptive immunological system confirms this idea. Human *crp/sap* might be coded by unique genes as a minimal remnant of phylogenetically earlier *crp*-like gene families which were more important when antibodies were not fully developed given their capacity to recognize a wide variety of ligands. Therefore, *crp*-like gene families might constitute an ancient link between innate and adaptive immune responses.

Further studies on how the zebrafish tCRP-like molecules and any of their transcript variants and/or protein conformations affect binding specificities and/or functionalities could help the understanding of immune evolution and improve prevention methods against diseases, including vaccines, adjuvants and other biotechnological tools. However, since most functional studies will have to use recombinant fish CRP-like proteins, caution with both LPS contamination (Pepys et al., 2005) and conformation composition should be carefully taken for a correct interpretation of future results.

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