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Short Communication

Molecular cloning and expression analysis of *Pleurodeles waltl* complement component C3 under normal physiological conditions and environmental stresses

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ABSTRACT

C3 is a component of the complement system that plays a central role in immunity, development and tissue regeneration. In this study, we isolated the C3 cDNA of the Iberian ribbed newt *Pleurodeles waltl*. This cDNA encodes a 1637 amino acid protein with an estimated molecular mass of 212.5 kDa. The deduced amino acid sequence showed that *P. waltl* C3 contains all the conserved domains known to be critical for C3 function. Quantitative real-time PCR (qRT-PCR) demonstrated that under normal physiological conditions, *P. waltl* C3 mRNA is expressed early during development because it is likely required for neurulation. Then, its expression increased as the immune system developed. In adults, the liver is the richest source of C3, though other tissues can also contribute. Further analysis of C3 expression demonstrated that C3 transcription increased when *P. waltl* larvae were exposed to pH or temperature stress, suggesting that environmental modifications might affect this animal's defenses against pathogens.

1. Introduction

The complement system is a critical component of innate immunity and is key in the protection against infections. This system involves a family of proteins that are mainly synthetized by the liver (Walport, 2001a,b), though lower levels can be produced by other cell types. Complement activation can be achieved in the extracellular space through three pathways (classical, lectins or alternative) and intracellularly *via* a cathepsin-dependent mechanism (Liszewski et al., 2013).

All complement activation pathways converge at the level of C3, the key component, which is processed into activation fragments C3a and C3b (Collard et al., 2000), with the assembly of C5 convertase and the further release of the C5a and C5b fragments. C3a and C5a are anaphylatoxins that exert a plethora of pro-inflammatory and immunoregulatory functions (Guo and Ward, 2005). In addition to their pro-inflammatory properties, these molecules regulate antigen-presenting cell functions and adaptive immune responses (Klos et al., 2009). Indeed, C3a and C5a have been shown to modulate the expression levels of MHC class II and costimulatory molecules on dendritic cells (Strainic et al., 2008; Weaver et al., 2010). C5b initiates the terminal phase of complement activation, *i.e.*, the formation of the membrane-attack complex (MAC) in association with the C6, C7, C8 and 1–18 molecules of C9, which leads to the formation of pores in pathogen membranes (Bhakdi and Tranum-Jensen, 1991; Esser, 1991).

In addition to their immunological functions, complement molecules are also involved in early vertebrate development. Indeed, the expression of complement molecules at the gastrula/early neurula stage and organ-specific expression patterns during organogenesis were observed in *Xenopus laevis* (McLin et al., 2008). Furthermore, a recent study showed that *Xenopus* neural crest cells are coattracted *via* the complement fragment C3a and its receptor C3aR (Carmona-Fontaine et al., 2011).

Complement also becomes activated during tissue injury and remodeling. Complement proteins have been shown to be involved in tissue and organ regeneration (Del Rio-Tsonis et al., 1998; Kimura et al., 2003; Mastellos et al., 2013). Distinct complement-triggered pathways have been shown to modulate critical responses that promote tissue reprogramming, pattern formation and regeneration across phylogenesis (Mastellos et al., 2013).

Despite these critical immunologic and nonimmunologic functions, complement molecules have only been considered in the context of regeneration studies in urodele amphibians (Del Rio-Tsonis et al., 1998; Kimura et al., 2003). Furthermore, no

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complete urodele mRNA complement sequence is available in the GenBank or EMBL databases, and, to our knowledge, no study has analyzed the expression of these important molecules in these species in response to environmental changes. Consequently, we characterized the C3 cDNA of the urodele amphibian *Pleurodeles waltl*, studied its tissue distribution and expression during embryonic development and the effects of pH and temperature changes on its expression.

2. Materials and methods

2.1. Animals and RNA extraction

P. waltl (embryos, larvae and adults) were reared in our animal facilities at 20 °C under controlled conditions approved by the French Ministry of Agriculture and Fisheries (agreement DDSV54/SPA/07/130) and treated in accordance with national legislation and the European Communities Council Directive on the Protection of Animals Used for Experimental and Other Scientific Purposes 86/609/EEC. To avoid as much as possible potential contamination, embryos and larvae were reared in Evian spring water (http://www.evian.com/files/evian%202013%20AWQR%20CALIF%20ENG.pdf).

Developmental stages were defined according to Gallien and Durocher (1957). Nine embryos or larvae were randomly chosen for each developmental stage studied and homogenized in TRIzol reagent (Invitrogen, Carlsbad, CA, USA) for RNA extraction. Total RNA was also isolated from the thymus of stage 44 (36-day-old) larvae and from various adult tissues (blood, brain, heart, intestine, kidney, liver, lung, spleen and testis).

2.2. pH and temperature challenge

To assess the effects of environmental changes on *P. waltl* C3 expression, larvae at stage 36–37 of development (14 days after laying, 2 days after hatching) were placed for 3 h in aquaria in which the pH was increased to 9 (initial pH was 7.2) or for 24 h in aquaria in which the temperature was 7 or 10 °C above the normal rearing temperature (20 °C). No larvae died during these two treatments. After 3 h at pH 9 or 24 h at 27 or 30 °C, the larvae were homogenized in TRIzol reagent for RNA extraction, except for one group that was sacrificed 14 h after the 27 °C heat shock. For each treatment, larvae of the same developmental stage reared under standard conditions were used as controls.

2.3. Cloning and sequencing of P. waltl C3 cDNA

One microgram of liver RNA was reverse transcribed using random primers and MMLV reverse transcriptase (Invitrogen, Carlsbad, CA, USA) in a final volume of 20 µl. Primers were designed in conserved regions identified by aligning C3 mRNA sequences from *Homo sapiens* (EMBL accession number BC150179), *Gallus gallus* (AAA64694), *Xenopus tropicalis* (XM_002940050), *Naja naja* (AAA49385), *Lethenteron camtschaticum* (AB377282), *Oncorhynchus mykiss* (L24433) and *Oryzias latipes* (AB025575). A first PCR reaction was performed with the C3-PW-For and C3-PW-Rev primers (Supplementary Table 1) using 10 ng of liver cDNA and Goldstar Taq polymerase (Eurogentec, Seraing, Belgium). The PCR program was as follows: 5 min at 94 °C, then 40 cycles of 30 s at 94 °C, 30 s at 55 °C and 1 min 30 s at 72 °C and finally 3 min at 72 °C. A single PCR product of 1316 bp was obtained, cloned in the pGEMT®-Easy vector (Promega, Madison, WI, USA) and sequenced. Next, specific primers (C3-PW-GSP1 and C3-PW-GSP2) and nested primers (C3-PW-NGSP1 and C3-PW-NGSP2) (Supplementary Table 1) were designed from the obtained sequence to perform 3′- and 5′-RACE using the BD SMART RACE cDNA Amplification kit (Clontech, Mountain View, USA) and MMLV reverse transcriptase (Invitrogen). The RACE products were cloned, sequenced and assembled to obtain the full-length *P. waltl* C3 cDNA.

2.4. Sequence analysis

Translation was performed using the Transeq program available on the portal for bioinformatic analyses of the Pasteur Institute (http://mobyle.pasteur.fr). Protein domains were determined *via* a BLAST analysis (http://blast.ncbi.nlm.nih.gov/). Sequence alignments were performed using ClustalW (http://www.ebi.ac.uk/Tools/msa/clustalw2/) and a phylogenetic tree was constructed by NJ method supported with 1000 bootstrap replications using the MEGA6 software (http://www.megasoftware.net/).

2.5. Real-time PCR

Primers specific for *P. waltl* C3, HSF1, HSP70, HSP90 and 4 housekeeping genes (GAPDH, α-actin, TAFII and mitochondrial 16S RNA) were designed using the Genscript software (http://genscript.com/ssl-bin/app/primer) (Supplementary Table 1) in different exons to ensure that they could not hybridize to potential traces of genomic DNA. The specificity of each primer pair was tested using a BLAST search through the U.S. National Center for Biotechnology Information (Bethesda, MD, USA). Real-time PCR was performed using the Mesa Green qPCR Master Mix (Eurogentec) and a Mastercycler® realplex^2 real-time PCR machine (Eppendorf, Hamburg, Germany). The cycling protocol was as follows: 3 min at 95 °C, followed by 40 cycles of 15 s at 94 °C and 30 s at the annealing temperature indicated in Supplementary Table 1. Standard curves were produced to ensure that the amplification efficiencies were similar and in the range of 95–105%. Melting curves were processed to check the quality of the PCR and verify the amplicon's melting temperature. Each PCR was performed in triplicate and repeated at least two times. Data were analyzed using the relative Pfaffl model (Pfaffl, 2001). Relative expression levels were calculated by comparison to the 4 housekeeping genes using the GeNorm software and Vandesompele's methodology (Vandesompele et al., 2002).

2.6. Western blotting

Proteins were prepared by lysing a pool of 3 larvae in lysis buffer containing 1% triton X-100, 150 mM NaCl, 10% glycerol, 0.1% SDS, 50 mM HEPES, 50 mM NaF, 1 mM NaVO3, 2 mM EDTA. Samples of 50 µg of protein were heated at 97 °C for 5 min, separated by SDS–PAGE and electrotransferred to a PVDF membrane (Amersham, Buckinghamshire, UK). Immunodetection was performed using antibodies against HSP70 (1:200) from Millipore (Billerica, MA, USA), HSP90 (1:5000) from Stressgen (Ann Arbor, MI, USA) or α-actin (1:20,000) from BD Transduction Laboratories (San Diego, CA, USA). Western blot results were quantified by densitometry using a Fusion Fx7 imaging system (Vilber Lourmat, Marne-la-Vallée, France) and expressions were normalized to α-actin.

2.7. Statistical analysis

Statistical analyses were performed using SPSS 13.0 (SPSS Inc., Chicago, IL, USA). Outlier values were determined by a boxplot of each group studied. When normality and homogeneity of variances were ascertained, Student *t* test analyses were performed to establish 2-group comparisons. Otherwise, Mann–Whitney tests were used for 2-group comparisons. Values of $p < 0.05$ and <0.10 were

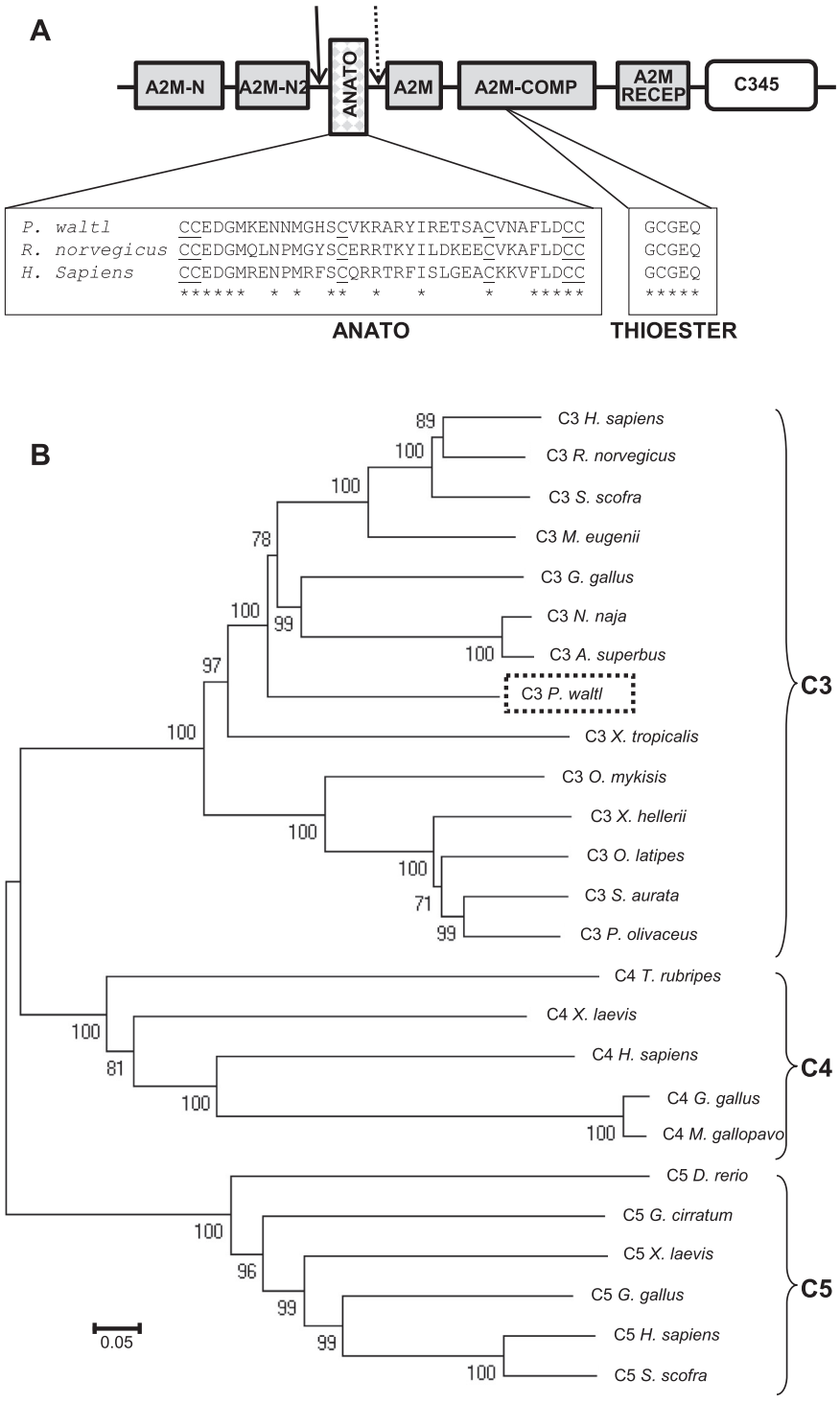

Fig. 1. *Pleurodeles waltl* C3 complement component. (A) Schematic representation of the *P. waltl* C3 protein. A2M-N = alpha2-macroglobulin N-terminus regions 1 and 2; ANATO = anaphylatoxin domain; A2M = alpha2-macroglobulin C-terminus region; A2M-COMP = alpha2-macroglobulin-like domain with the thioester domain; A2M-RECEP = alpha2-macroglobulin receptor domain; C345 = complement C3/4/5 C-terminal region. The cleavage site that limits the α and β chains of C3 and the C3 convertase cleavage site are indicated by plain and dashed arrows, respectively. Note that the boxes are not to scale. The anaphylatoxin and thioester domains were aligned with the corresponding rat and human regions to show their conservation. The six canonical cysteine residues located at conserved positions and known to be critical for the function of the ANATO domain are underlined. * Indicates identical residues. (B) Phylogenetic tree constructed from the amino acid sequences of C3, C4 and C5 molecules of various species. Amino acid sequences were aligned using ClustalW and the tree was constructed by NJ method supported with 1000 bootstrap replications using the MEGA6 software. Bootstrap values are indicated. The scale bar corresponds to the evolutionary distance. The following sequences were used: C3 – *Homo sapiens* (BC150179), *Rattus norvegicus* (NM_016994), *Sus scofra* (NM_214009), *Macropus eugenii* (AY746459), *Gallus gallus* (AAA64694), *Naja naja* (AAA49385), *Austrelaps superbus* (DQ149984), *Pleurodeles waltl* (KJ619514), *Xenopus tropicalis* (XM_002940050), *Oncorhynchus mykiss* (L24433), *Xiphophorus hellerii* (AEJ08067), *Oryzias latipes* (AB025575), *Sparus aurata* (HM543456), *Paralichthys olivaceus* (BAA88901); C4 – *Takifugu rubripes* (CAD45003), *Xenopus laevis* (NM_001087465), *Homo sapiens* (NM_001252204), *Gallus gallus* (NM_001077233), *Meleagris gallopavo* (ACA64782); C5 – *Homo sapiens* (NM_001735), *Sus scofra* (AY332748), *Gallus gallus* (XM_415405), *Xenopus laevis* (NM_001204059), *Ginglymostoma cirratum* (EU797190), *Danio rerio* (XP_001919226).

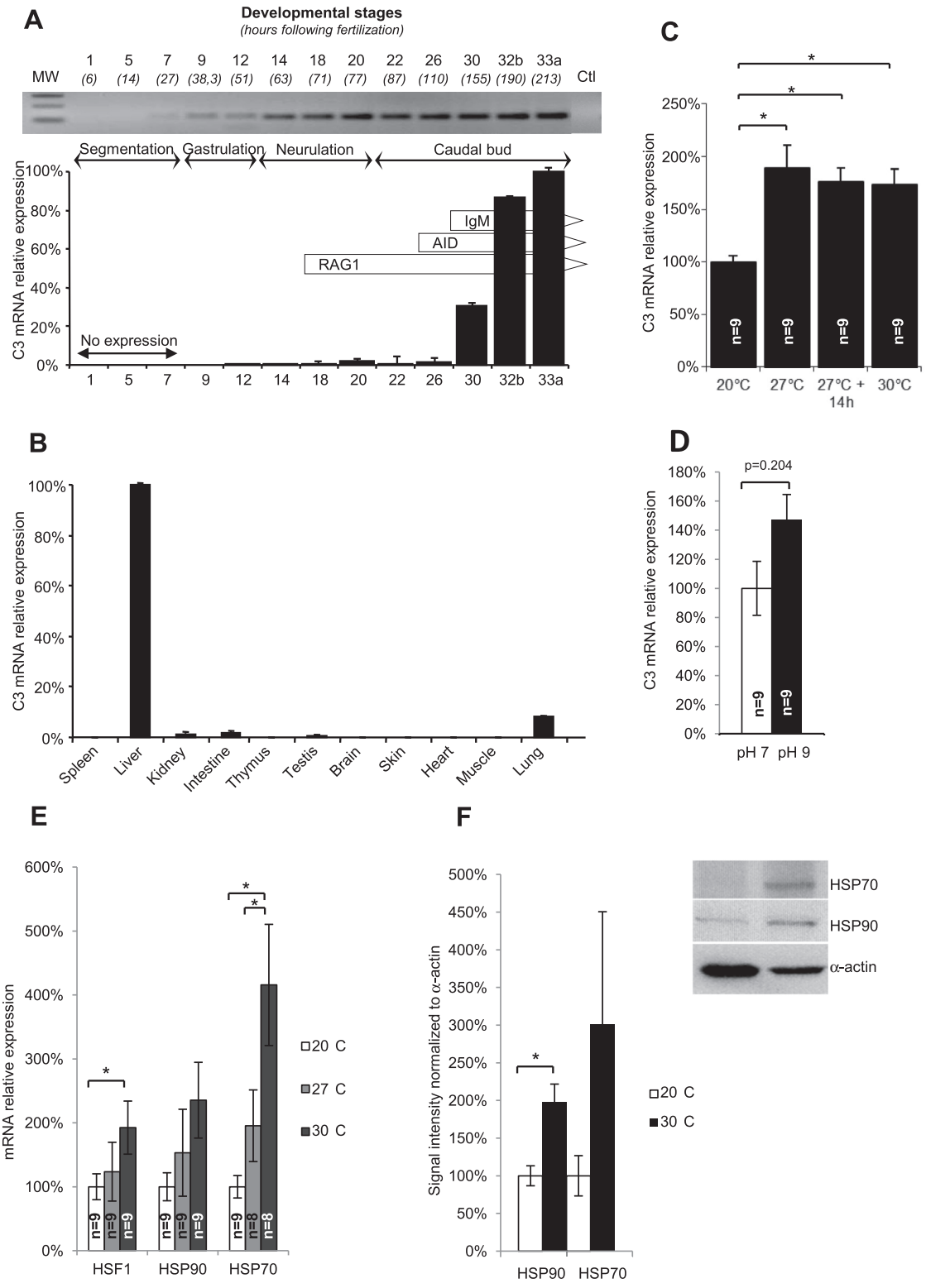

Fig. 2. *P. waltl* C3 mRNA expression profiles. (A) C3 mRNA expression during early stages of *P. waltl* development. The developmental stages and corresponding hours after fertilization are indicated at the top of the figure. qPCR products separated on an agarose gel are shown. MW = molecular weight. Ctl = negative control. The qPCR results are expressed as a percentage of the signal obtained with stage 33a larvae. The horizontal arrows indicate the expression of AID, IgM heavy chain and RAG1 transcripts, as reported in previous studies (Frippiat et al., 2001; Boudarra et al., 2002; Bascove and Frippiat, 2010). (B) Expression of C3 mRNAs in various *P. waltl* tissues. The relative value obtained with the liver was set to 100%. (C) Quantification of C3 transcripts in larvae exposed during 24 h to heat shocks or during 3 h to a pH increase (D). The relative value obtained with control larvae was set to 100%. (E) Quantification of HSF1, HSP70 and HSP90 mRNAs in larvae exposed to 27 or 30 °C heat shocks and controls reared at 20 °C. The relative value obtained with control larvae was set to 100%. (F) Quantification of HSP proteins confirmed qPCR results. Western blot results were analyzed and quantified by densitometry using a Fusion Fx7 imaging system (Vilber Lourmat, Marne-la-Vallée, France) and HSP expressions were normalized to α-actin. Representative western blot results are shown (right panel). Each lane contains proteins from 3 larvae. These experiments were repeated at least 3 times * indicates statistically significant differences ($p < 0.05$). For each panel, the values represent the means ± SEM.

selected to indicate significance and trend, respectively. All the results are shown as means ± SEM.

3. Results and discussion

3.1. Characteristics of P. waltl C3 cDNA

Overlapping 5′- and 3′-RACE PCR products were assembled to obtain the full-length *P. waltl* C3 cDNA sequence, which was deposited in GenBank (accession number KJ619514). This cDNA has an open reading frame of 4911 bp and encodes a 1637 amino acid protein with an estimated molecular mass of 212.5 kDa. In contrast to Lambris et al. (1995) who described an *X. laevis* alternative C3 transcript that lacks many of the ligand binding sites, no splice variant was detected during this study.

Alignment of the predicted *P. waltl* C3 amino acid sequence with C3 sequences from other species showed that this molecule possesses all the domains typical of C3 complement molecules. Indeed, our sequence possesses five α2-macroglobulin domains (C3, C4 and C5 are members of the α2-macroglobulin family), one anaphylatoxin domain responsible for inflammatory activities and the complement C3/4/5 C-terminal region (Fig. 1A). Moreover, *P. waltl* C3 possesses the highly conserved cleavage site RXXR (RRRR for *P. waltl*) at residues 662–665 upstream of the anaphylatoxin domain, which defines the limit between the α and β chains of C3 (plain vertical arrow in Fig. 1A), as in all currently known C3 sequences. The anaphylatoxin domain is located at the N-terminus of the α chain and contains all the residues known to be critical for its functions (Castillo et al., 2009; Qi et al., 2011). The conserved cleavage site recognized by C3 convertase is also present in the predicted C3 sequence (LFLARSEVEE, cleavage between the R and S residues, as indicated by the dashed vertical arrow in Fig. 1A) at residues 737–746, leading to a predicted *P. waltl* C3a fragment of 76 amino acids (78 amino acids in human and mouse) containing the anaphylatoxin domain. Finally, the thioester motif GCGEQ, allowing the covalent binding of C3 to the target cell membrane (Law and Dodds, 1997), is present at residues 1003–1007 in the predicted *P. waltl* C3 amino acid sequence.

3.2. Phylogenetic analysis

To confirm that we isolated the C3 cDNA of *P. waltl*, we built a phylogenetic tree with C3, C4 and C5 sequences (Fig. 1B). This tree shows that the sequence identified in *P. waltl* clusters with C3 proteins and not with C4 or C5 sequences. *P. waltl* C3 presents between 53% and 61% similarity with the C3 proteins used to construct this tree, the highest similarities (61%) being found with amino acid sequences from *Austrelaps superbus* and *N. naja*.

3.3. Expression profile during embryonic development and tissue distribution

The expression patterns of C3 during embryogenesis and in *P. waltl* tissues were analyzed by qRT-PCR. As shown in Fig. 2A, the first expression of C3 was detected at stage 7 and remained low during gastrulation. C3 transcription increased during neurulation (from stage 14 to 20) and was stronger after stage 26 when immunoglobulin heavy chain transcripts are expressed. Thus C3 mRNA expression preceded the expression of other immunological genes (RAG1, AID and IgM heavy chains) (Frippiat et al., 2001; Boudarra et al., 2002; Bascove and Frippiat, 2010). Similarly, C3 transcripts were detected at the gastrula/early neurula stages in *X. laevis* (McLin et al., 2008) and were shown to be required for the proper migration of neural crest cells (Carmona-Fontaine et al., 2011). Thus, as in *X. laevis*, C3 is likely expressed at early developmental stages in *P. waltl* because it is required for neurulation. Fig. 2B shows that, as in other vertebrates, that the liver is the major source of complement C3 expression. Some lower levels of transcription could be detected in the lungs, intestine, kidney and testis.

Taken together, the sequence and expression features suggest that *P. waltl* C3 functions in a similar way to the C3 molecules of other animals.

3.4. Expression after a pH or temperature challenge

Environmental parameters affect the physiological responses of aquatic animals (Bascove et al., 2011; Qi et al., 2011; Frippiat, 2013; Huin-Schohn et al., 2013; Schenten et al., 2013). Consequently, we investigated the expression profile of C3 in *P. waltl* larvae exposed to temperature or pH stress. Our data reveal a significant increase in C3 mRNA expression following exposure to two heat shocks (27 or 30 °C) for 24 h (Fig. 2C) and an increase, which was not statistically significant, in C3 mRNA expression following exposure to pH 9 for 3 h (Fig. 2D). We also noted that the temperature-induced increase in transcription was rather stable because the same level of C3 mRNA was still observed 14 h after the end of the 27 °C heat shock. These responses could be mounted by *P. waltl* larvae to neutralize harmful free radicals generated by stress treatments. Indeed, previous studies have shown that pH and temperature challenges can cause oxidative damage and the generation of ROS in ark shell, Pacific white shrimp, Mediterranean mussel and orange-spotted grouper (Frenzilli et al., 2001; Wang et al., 2009; An and Choi, 2010; Qi et al., 2011). Furthermore, it has been shown that complement activation plays an important role in the inflammatory process after oxidative stress (Collard et al., 1999). There is increasing evidence that ROS activate the complement system and C3 is thought to provide protection against oxidative stress (Collard et al., 1999, 2000; Hart et al., 2004). Another possibility is that danger signals, such as HSP, that are induced by heat shock (Fig. 2E) up-regulate C3 expression, as shown in photodynamic therapy-treated tumors (Stott and Korbelik, 2007). An inspection of know C3 promoter sequences (human, mouse and rat) using the Match and TFSEARCH programs (available at http://www.gene-regulation.com/cgi-bin/pub/programs/match/bin/match.cgi and http://www.cbrc.jp/research/db/TFSEARCH.html) confirmed the presence of heat shock protein binding sites (data not shown). However, verifying this hypothesis will require the cloning and analysis of the *P. waltl* C3 promoter to determine whether it also contains heat shock response element binding sites.

In conclusion, we cloned the C3 cDNA from *P. waltl* for the first time and showed the conservation of this molecule in urodeles. This molecule is expressed early during development because it is likely required for neurulation. Then, its expression increased as the immune system developed. As in other vertebrates, the liver is the richest source of C3 in adults. Furthermore, we observed increased C3 mRNA levels after the exposure to temperature or pH increases, suggesting an activation of the immune system of this species.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.dci.2014.04.011.

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