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c-Raf participates in adaptive immune response of Nile tilapia via regulating lymphocyte activation

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ABSTRACT

RAF proto-oncogene serine/threonine-protein kinase (c-Raf) is a MAP kinase kinase kinase (MAPKKK) that participates in the Erk1/2 pathway and plays an important role in lymphocyte activation. However, the study on how c-Raf regulates adaptive immunity in non-mammal is still limited. In present study, based on analysis of sequence characteristics of c-Raf from *Oreochromis niloticus* (On-c-Raf), we investigated its regulation roles on teleost lymphocyte activation. The On-c-Raf was highly conserved during evolution, which was composed of a Raf-like Ras-binding domain (RBD), a protein kinase C conserved region 1 (C1) domain and a serine/threonine protein kinase catalytic (S_TKc) domain. Its mRNA showed a wide distribution in tissues of *O. niloticus* and with the highest expression in gill. After *Aeromonas hydrophila* infection, during the adaptive immune stage transcription level of On-c-Raf was significantly upregulated on day 8, but came back to original level on day 16 and 30, suggesting the potential involvement of On-c-Raf in primary response but not memory formation. Furthermore, On-c-Raf mRNA in leukocytes of Nile tilapias was obviously induced by in vitro stimulation of T cell mitogen PHA. More importantly, in vitro stimulation of lymphocytes agonist PMA augmented phosphorylation level of On-c-Raf in leukocytes detected by western-blot and immunofluorescent. Thus, c-Raf regulated lymphocyte activation of Nile tilapia on both mRNA and phosphorylation level. Together, our results revealed that the c-Raf from teleost Nile tilapia engaged in adaptive immune response by regulating lymphocytes activation. Since the regulatory mechanism of lymphocyte-mediated adaptive immunity is largely unknown in teleost, our study provided important evidences to understand teleost adaptive immunity, and also shed a novel perspective for the evolution of adaptive immune system.

1. Introduction

The protein kinase family, which plays a predominant role in almost every cellular biology [1] by catalyzing phosphorylation of downstream molecules, is one of the largest gene families [2]. Among them, the mammalian Raf kinase family consists of A-Raf and B-Raf and c-Raf (or Raf 1). The first Raf gene was isolated from murine retrovirus in 1983, which was named Virus-induced Rapidly Accelerated Fibrosarcoma (V-RAF) at that time because of its ability to transform rodent fibroblasts to cancer cells [3]. Then the v-Raf was proven to be an enzyme demonstrating serine/threonine-specific protein kinase activity in the next year [4]. Later the cellular Raf (c-Raf) with homologous functions was found in mouse [5], and increasing studies were carried out making us comprehensively understand this molecule.

The c-Raf has been proven an upstream activator of mitogen-activated protein kinase (MAPK), which is an important group of mediators

transducing signals from cell surface to nucleus under stimuli including cytokines, growth factors, exogenous pressure and mitogens [6]. As a firstly discovered MAPK cascade, the Ras-Raf-Mek-Erk pathway has been proved to ubiquitously engage in various basic cell functions such as proliferation, differentiation, survival, apoptosis and gene expression [6,7]. For example, the *c-raf*-knockout mouse embryos could not survive despite the activating MAPK cascade [8], indicating its indispensable role. The RAF was also proven to be an oncogenic gene that was closely associated with tumorigenesis. Expressing the kinase domain of c-Raf led to lung adenomas in transgenic mice [9]. Furthermore, the level of c-Raf was upregulated in human glioblastoma multiforme and germ line mutation of CRAF induced leukemia [10,11].

The c-Raf also plays an essential role in the regulation of lymphocytes-mediated adaptive immunity [12,13]. As a MAPKKK, c-Raf is the initiator of the MAPK tertiary cascade. When TCR signaling initiates, the c-Raf is activated by Ras through Zap-70-PLC γ 1-RasGRP and Zap-

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70-LAT-SOS pathway, which ultimately activates Erk [14–17]. The activation of Erk was once reported to promote the positive selection during T cell development [18], and c-Raf might contribute to the process of mutant thymocytes as well in a recent study [19]. The previous study also showed that Raf-CAAX could transformed thymocytes from double-negative to double-positive [20]. In a Raf-transgenic LATY136F mutation mouse model, constitutive expression of activated Raf could enhanced the MAPK cascade, resulting in a pathologic uncontrolled proliferation of T lymphocytes that characterized by an oversize spleen [19]. In addition to T lymphocytes, c-Raf also participates in the activation and function of B lymphocytes via PLC γ 2-RasGRP-c-Raf-Erk axis that activates Bcl-6 and Egr1 transcription factors [21,22].

As an important weapon of adaptive immunity, the regulation of lymphocyte-mediated adaptive immunity has been well elucidated in mammals. However, the regulatory mechanism of adaptive immunity remains unclear in lower vertebrates, especially in non-tetrapod. Recently, several suspected T cell subsets have been identified from teleost, such as CD3⁺, CD4-1⁺, CD4-2⁺ or CD8⁺ lymphocytes [23–30]. Meanwhile, these subsets of lymphocytes performed similar functions with that of mammals, such as antigen-specific proliferation, graft-versus-host reaction (GVHR), cell-mediated cytotoxicity (CMC), antibacterial responses, tumor infiltration and antigen presentation [24,26,27,31,32], demonstrating that T cell-mediated adaptive immune response indeed exist in teleost. On the other hand, B cells that express IgM, IgD or IgT have been identified from several kinds of teleost and play critical roles in adaptive immunity of fish [33–35]. Nevertheless, the regulatory mechanism of lymphocytes-mediated adaptive immunity in teleost is still unknown, which needs to be further understood. In present study, we analyze the sequence properties of a c-Raf from Nile tilapia *Oreochromis niloticus*, investigate its tissue expression pattern, and unveil its regulation on mRNA and phosphorylation level upon lymphocyte activation, hoping to shed light to regulatory mechanism of the adaptive immune system of teleost.

2. Materials and methods

2.1. Experimental animals

Nile tilapia *Oreochromis niloticus* ordered from an aquatic farm in Guangzhou, Guangdong Province, China, were raised in aerated fresh water at 28 °C. Fish were fed with commercial pellets daily, and health fish with body length of 8–10 cm were used for experiments.

2.2. Sequence and structural analyses

We obtained the cDNA and amino acid sequence of c-Raf in *O. niloticus* (accession number: XM_003438987 and XP_003439035) from NCBI. The multiple sequence alignment was performed with the software ClustalX 1.83 and displayed by the sequence manipulation suite (SMS). We constructed the phylogenetic tree with MEGA4 through neighbor joining (NJ) algorithm. We predicted the protein domains by the simple modular architecture research tool (SMART) and the functional sites by Prosite, respectively. We established the tertiary structures of proteins with SWISS-MODEL prediction algorithm [36,37] and displayed them by PyMOL software.

2.3. mRNA expression pattern of On-c-raf in different tissues

The total RNA of blood, liver, spleen, gill, head kidney and trunk kidney were extracted from four healthy Nile tilapias as parallel samples with TRIzol reagent (Invitrogen). The RNA was then treated with DNase at 37 °C for 30 min to degrade DNA, and at 65 °C for 10 min to stop reaction. The treated RNA was used as template with oligo (dT) -adapter as primer to synthesize the first strand of cDNA using M-MLV reverse transcriptase. The cDNA product was diluted for SYBR Green

fluorescent real-time quantitative PCR (RT-PCR).

The pair of gene-specific primers (TGAAAAGTTGGTGGCTGACTG and CGGTTTATCTTAGGGAGGGCAT) was synthesized for On-c-Raf to amplify a 112 bp product in RT-PCR. The β -actin was selected as internal control to normalize the templates for corresponding tissue samples with two primers (CGGAATCCACGAAACCACCTA and CCAGACGGAGTATTACGCTCA). The RT-PCR was implemented with CFX Connect Real-Time System (BIO-RAD). The expression level of On-c-Raf was analyzed by comparative Ct method ($2^{-\Delta\Delta C_t}$ method) [38].

2.4. Expression of On-c-raf after bacterial stimulation

Healthy Nile tilapias were intraperitoneally injected with 100 μ L 3.6×10^6 CFU ml⁻¹ *Aeromonas hydrophila* suspension as challenge group, and with PBS as control group. Each group was randomly sampled at 5th, 8th, 16th and 30th day post stimulation. The spleens were separated and prepared for RT-PCR assay as aforementioned protocols to detect the mRNA level of On-c-Raf.

2.5. Isolation of leukocytes

Nile tilapia leukocytes were isolated from spleen according to previous report with slight modification [29,33,39]. Briefly, spleen was smashed in Leibovitz's L-15 medium (Gibco) containing 2% FBS and collected through a cell filter membrane. The cell suspension was centrifuged at 800 g, 4 °C for 5 min and the cell pellet was resuspended in 3 mL L-15 medium containing 2% FBS prepared on ice. The leukocytes were separated by Percoll density gradient centrifugation. The pure Percoll (GE Healthcare) was 9:1 mixed with $10 \times$ PBS (15 mM K₂HPO₄, 80 mM NaH₂PO₄·12H₂O, 30 mM KCl and 1.4 M NaCl) to 100% Percoll, and then diluted with L-15 medium to 34% and 52% (v/v) Percoll respectively. The diluted Percoll was pre-heated to room temperature and hierarchically added to a 15 mL centrifuge tube (5 mL each, 34% for superstratum and 52% for substratum). The cell suspension was gently added to the top and centrifuged at 500 g, room temperature for 30 min with the lowest acceleration and deceleration. The cells between 34% and 52% Percoll were collected, washed twice with L-15 medium for subsequent experiments.

2.6. Expression pattern of On-c-raf after PHA stimulation

1×10^7 leukocytes isolated from spleen were stimulated with 10μ g mL⁻¹ phytohaemagglutinin (PHA, Sigma) in DMEM containing 10% FBS and 1% penicillin/streptomycin, and cultured at 28 °C with 5% CO₂. The leukocytes were collected at 5 h and 10 h post stimulation respectively and unstimulated leukocytes were used as control. The samples were used for RT-PCR assay to quantify the mRNA level of On-c-Raf as described above.

2.7. Western-blot assay of On-c-raf phosphorylation after PMA stimulation

The spleen leukocytes that harvested from Nile tilapias as described above were resuspended in 500 μ L D-PBS (with Ca²⁺ and Mg²⁺, BBI Life Sciences), rested at 28 °C for 30 min to remove innate phosphorylation and treated with 50 ng mL⁻¹ Phorbol 12-myristate 13-acetate (PMA, Sigma). Then the stimulated leukocytes were respectively collected at 10 and 30 min and added 500 μ L pre-cooled D-PBS to terminate stimulation. The resting leukocytes were used as control. After spun down, the cells were resuspended in NP40 (Sigma) lysis buffer on ice for 30 min and centrifuged at 13000 g, 4 °C for 10 min to gain supernatant. The relative protein quantity of cell lysate was normalized by Quick Start Bradford 1 $\times$ Dye Reagent (Bio-rad) method.

The protein samples were separated by SDS-PAGE and transferred onto a nitrocellulose (NC) membrane in a waterish electrophoretic transfer system at 100 V for 2 h. The membrane was blocked with 4% non-fat powdered milk at room temperature for 1 h and incubated with

1:1000 diluted rabbit anti-phospho-c-Raf antibody (Ser338, Cell Signaling Technology) or rabbit anti- β -actin antibody (Cell Signaling Technology) in 2% bovine serum albumin (BSA, BBI Life Sciences) overnight at 4 °C, respectively. After three rinses with PBST (PBS with 0.05% Tween-20), the membrane was incubated with goat anti-rabbit IgG H&L Alexa Fluor 790 (Abcam) 1:10000 diluted in 4% non-fat powdered milk at room temperature for 1 h and observed by Odyssey CLx Image Studio after another three rinses. The β -actin was selected as internal control.

2.8. Immunofluorescence assay of On-c-raf phosphorylation after PMA stimulation

The splenic leukocytes of Nile tilapias were collected and divided into two groups: one was rested as control and the other was treated with PMA for 15 min, following the protocol described above. The processed leukocytes were spun onto slides with Cytospin, and fixed in methyl alcohol for 5 min. The fixed slides were blocked with 1% BSA for 1 h at room temperature. The cells were then successively incubated with rabbit anti-phospho-c-Raf antibody (Ser338, Cell Signaling Technology, 1:200) and mouse anti- β -actin antibody (Cell Signaling Technology, 1:1000), and followed with Alexa Fluor 488-conjugated goat anti-rabbit IgG (H + L) (1:1000) and 594-conjugated goat anti-mouse IgG (H + L) (1:1000) as secondary antibodies respectively in a moist chamber for 1 h at 37 °C. After each incubation, a thrice rinse with PBST was performed. The slides were mounted with sealing glycerol containing DAPI and finally observed with a fluorescence microscope.

2.9. Statistical analysis

All data were displayed in terms of mean $\pm$ SE (n = 4). The two-tails *t*-test was applied to define the statistical significance and the *p* value less than 0.05 was labeled with * above the bars in figures.

3. Results

3.1. Sequence characteristics of On-c-raf

The On-c-Raf protein consists of 633 amino acids encoded by a 3058 bp mRNA. The multiple sequence analysis showed that the amino acid sequence of On-c-Raf was well conserved compared to those of the other seven selected sequences, ranging from fruit fly to human (Fig. 1). Multitude of phosphorylation sites in On-c-Raf were conserved compared to those in most selected species, including S50, S64, S273, S303, S348, Y351, S481, T501 and S631, only with exception on S301 which was substituted by valine in On-c-Raf (Fig. 1). Besides, the cysteine-rich C1 domain, as well as the putative active site D478, the ATP-binding region IGSGSFGV (I365 to V373) and K385 were also well conserved (Fig. 1).

3.2. Structural characteristics of On-c-raf

According to SMART prediction, the On-c-Raf contains multiple domains including a Raf-like Ras-binding domain (RBD), a protein kinase C conserved region 1 (C1) domain and a serine/threonine protein kinase catalytic (S_TKc) domain, which was similar to c-Rafs from fruit fly and human (Dm-c-Raf and Hs-c-Raf) (Fig. 2a). However, v-Raf, the firstly discovered Raf protein, only contains a kinase domain (Fig. 2a). The kinase domains of On-c-Raf and the other three Rafs were employed for homologous modeling by SWISS-MODEL. Their tertiary structures were highly similar. The On-c-Raf was mainly composed of nine α -helices and eight β -strands linked by loops (Fig. 2b). Furthermore, the relative position of the ATP-binding region (I365 to V373 and K385), which was constituted of a strand-loop-strand motif and a lysine located on the other strand, and proton acceptor D478 were highly

conserved (Figs. 1 and 2b). Interestingly, On-c-Raf could dimerize like Hs-c-Raf while v-Raf and Dm-c-Raf only existed in monomers predicted by the SWISS-MODEL (Fig. 2b).

3.3. Phylogenetic characteristics of On-c-raf

The whole amino acid sequences of On-c-Raf and thirty other c-Rafs were used to construct the unrooted cladogram employing neighbor joining algorithm based on multiple sequence alignment. The On-c-Raf was firstly clustered with c-Rafs from *Pundamilia nyererei* and *Maylandia zebra*, and then clustered to those from the other four bony fishes, which suggested a close relationship of c-Rafs in teleost. The teleost group formed sister clusters to amphibian, avian, reptile and mammal in vertebrates, while invertebrate c-Rafs from arthropoda and nemathelminthes were distinctively clustered (Fig. 3).

3.4. The expression pattern of On-c-raf in tissues

To investigate the potential function of On-c-Raf in adaptive immunity, mRNA expression pattern was examined in the lymphoid related tissue by real-time RT-PCR. In healthy Nile tilapias, the On-c-Raf was expressed in a wide range of tissues with various levels including liver, head kidney, spleen, trunk kidney, blood and gill, among which the expression of On-c-Raf was extremely high in gill (Fig. 4). The relative mRNA levels of On-c-Raf in trunk kidney and blood were also high, which were about 3- and 4-fold compared to that in liver, while the expression levels were lower in spleen and head kidney and lowest in liver (Fig. 4).

3.5. Inducible expression of On-c-raf during adaptive immunity against bacterial infection

To figure out whether On-c-Raf was involved in adaptive immune response, we infected animals with pathogen *A. hydrophila*. The mRNA expression level of On-c-Raf in spleen was monitored by RT-PCR. Although transcriptional level of On-c-Raf in infected fish was comparable with control group 5 days after infection, it significantly up-regulated to more than 2.5-fold compared to PBS control 8 days after infection, however, the mRNA expression come back to basal level on 16 and 30 days after infection (Fig. 5). This expression pattern suggested c-Raf might participate in the primary response but not memory formation during adaptive immune response of Nile tilapia.

3.6. mRNA regulation of On-c-raf during lymphocyte activation

Since On-c-Raf was involved in adaptive immunity, we further explored the possible regulatory mechanism by stimulating the leukocytes with T cell mitogen PHA. The mRNA expression level of On-c-Raf significantly increased 10 h but not 5 h after PHA stimulation compared to unstimulated control (Fig. 6a), indicating that On-c-Raf participated in lymphocyte activation.

3.7. Phosphorylation of On-c-raf upon lymphocyte activation

To further rule out the regulatory role of On-c-Raf on lymphocyte activation, we stimulated the leukocytes with lymphocyte activation agonist PMA. The phosphorylation of On-c-Raf (p-c-Raf) was examined by western-blot and immunofluorescence assay. The p-c-Raf promptly responded to PMA stimulation and it was obviously enhanced at 10 min and 30 min post stimulation (Fig. 6b). Meanwhile, the visible On-c-Raf phosphorylation was also detected in cytoplasm of PMA-stimulated leukocytes, revealing by green fluorescence signal (Fig. 6c). As control, little positive signal could be observed in rested leukocytes (Fig. 6c). Thus, Nile tilapia c-Raf regulated activation of lymphocytes through increasing phosphorylation.

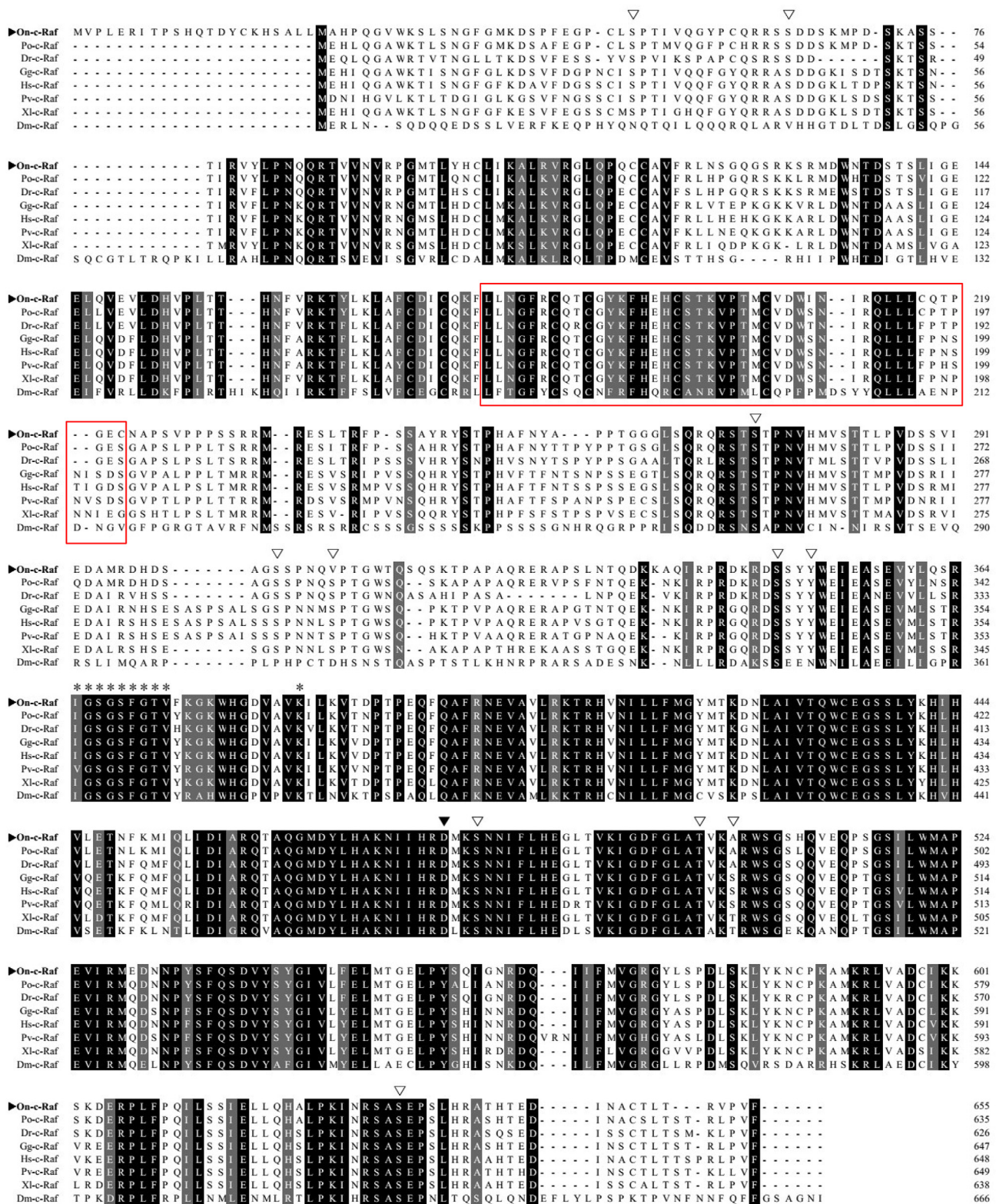


Fig. 1. Multiple sequence alignment of On-c-Raf with its homologues from other species. The amino acid residues colored in black are conserved in 100% sequences and similar residues are shaded in gray. The conserved active site, phosphorylation sites, ATP-binding region and C1 domain are marked with ▼, ▽, * and red boxes, respectively. The chosen homologues are from *O. niloticus* (On-c-Raf, XP_003439035), *Paralichthys olivaceus* (Po-c-Raf, XP_019951823), *D. melanogaster* (Dm-c-Raf, CAA30166), *Xenopus laevis* (Xl-c-Raf, NP_001081475), *Gallus gallus* (Gg-c-Raf, NP_990638), *Pogona vitticeps* (Pv-c-Raf, XP_020658205), *Danio rerio* (Dr-c-Raf, XP_017208994), and *H. sapiens* (Hs-c-Raf, NP_001341619). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

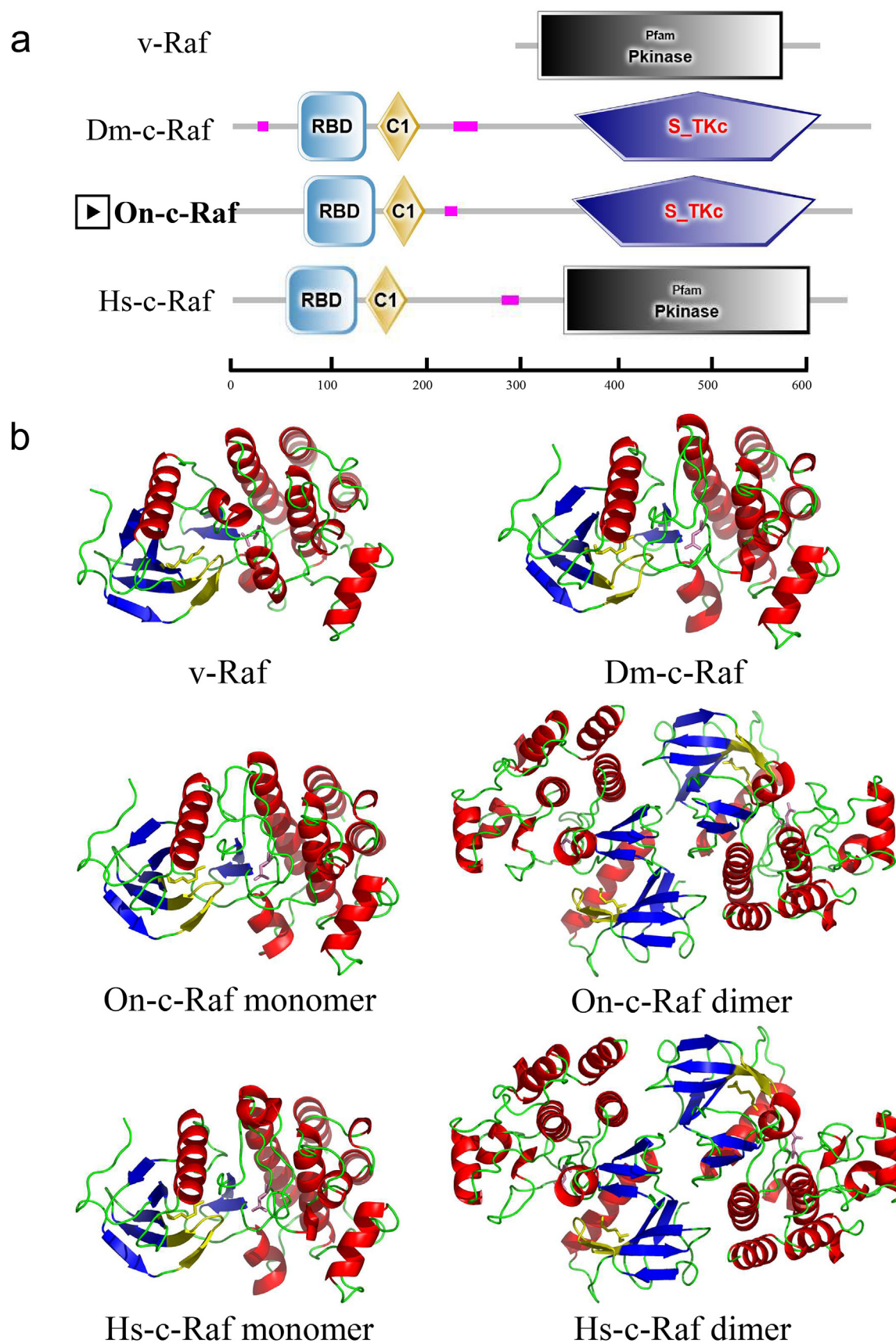


Fig. 2. Predicted structures of Rafs. (a) Predicted domains of full-length Rafs. The domains Pkinase, RBD, C1 and S_TKc represents protein kinase domain, Raf-like Ras-binding domain, protein kinase C conserved region 1 domain and Serine/Threonine protein kinase catalytic domain. (b) Predicted tertiary structures of Raf protein kinase domains. Different colors are defined as: red, α -helix; blue, β -strand; green, random coil; yellow, ATP-binding region; pink, active site, shown in stick model. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

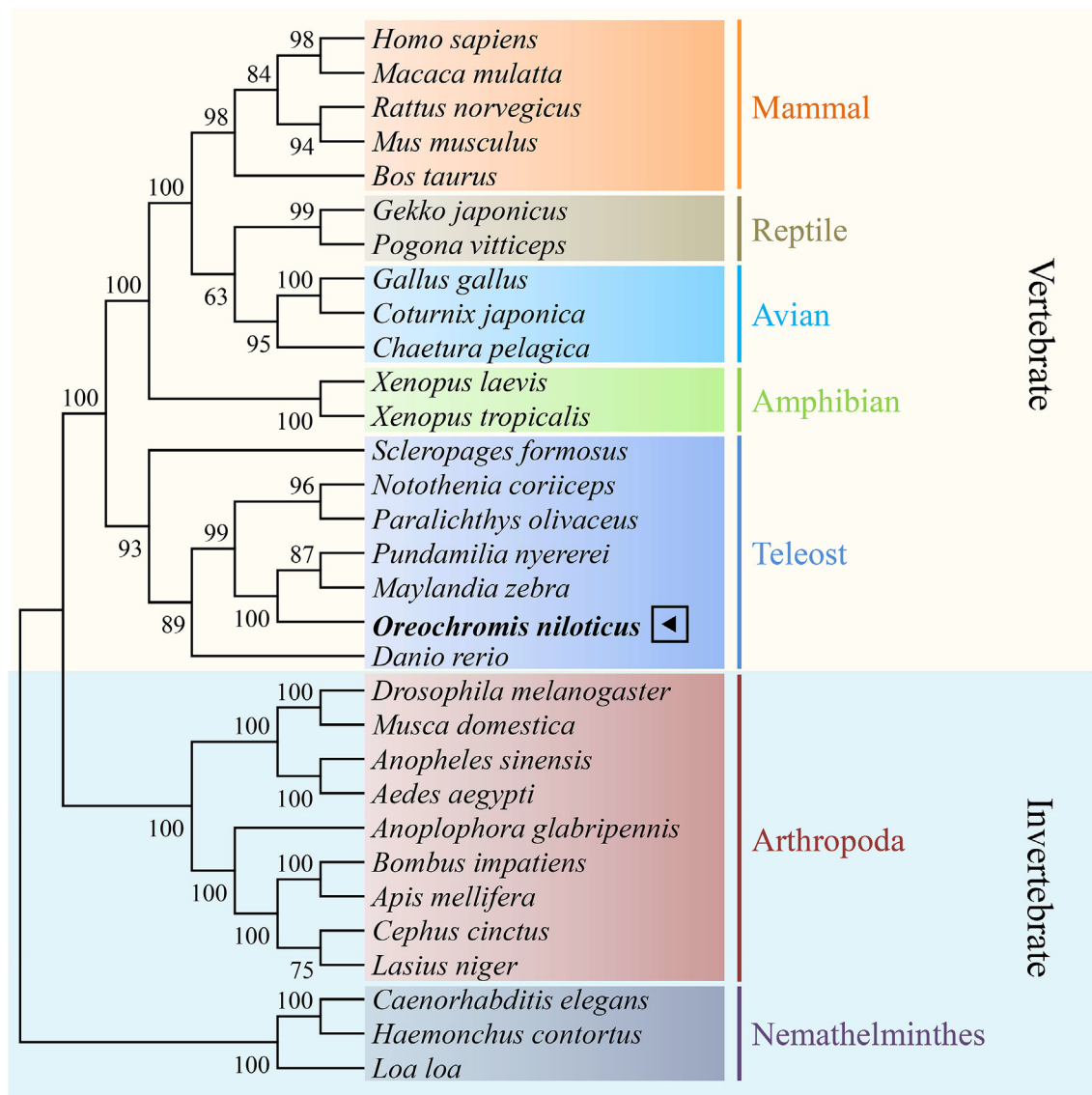


Fig. 3. Phylogenetic tree of On-c-Raf with its homologues. The protein sequences employed in the tree include c-Rafs from *O. niloticus* (XP_003439035), *P. olivaceus* (XP_019951823), *D. melanogaster* (CAA30166), *X. laevis* (NP_001081475), *G. gallus* (NP_990638), *P. vitticeps* (XP_020658205), *D. rerio* (XP_017208994), *H. sapiens* (NP_001341619), *Mus musculus* (NP_001343263), *Rattus norvegicus* (NP_036771), *Bos Taurus* (XP_015324294), *Macaca mulatta* (NP_001253231), *Caenorhabditis elegans* (CCD62343), *Musca domestica* (XP_019893780), *Aedes aegypti* (XP_021710700), *Anopheles sinensis* (KFB43995), *Lasius niger* (KM93735), *Cephus cinctus* (XP_015601956), *Bombus impatiens* (XP_024224515), *Loa loa* (XP_020306396), *Haemonchus contortus* (CDJ87890), *Apis mellifera* (XP_396892), *Gekko japonicus* (XP_015275732), *P. vitticeps* (XP_020658205), *Xenopus tropicalis* (NP_001017117), *Chaetura pelagica* (XP_010007148), *Coturnix japonica* (XP_015730206), *M. zebra* (XP_004546233), *P. nyererei* (XP_005749099), *Notothenia coriiceps* (XP_010784825), and *Scleropages formosus* (XP_018593102). Bootstrap values of 1000 replicates (%) are indicated for the branches.

4. Discussion

The c-Raf, which plays an essential role in MAPK cascade, participates in numerous cellular processes [40]. Although the structure and functions of c-Raf has been well studied in mammals, the related studies in teleost are still limited. In the present study, the c-Raf from *O. niloticus* (On-c-Raf) has been proven highly conserved compared to those from higher animals. The On-c-Raf also participates in adaptive immune response and regulates lymphocyte activation. For the first time, our study provides valuable knowledge about the regulatory mechanism in bony fish.

The amino acid sequence of On-c-Raf was highly similar with c-Rafs from other species based on multiple sequence alignment, especially the phosphorylation sites and functional regions. As a MAPKKK initiating the cascade, the activity of Raf is precisely regulated owing to its multidomain, which contains three conserved regions (CRs): 1) CR1, a

Ras-binding domain (RBD) and a Cys-rich domain (CRD); 2) CR2, a Ser/Thr-rich domain; 3) CR3, a protein kinase domain [41]. The c-Raf is a kinase whose activity can be regulated by phosphorylation on serine or threonine and sometimes on tyrosine [42–45]. Ser259 and Ser621 are the most predominant negatively regulatory sites of human c-Raf, which can be combined by the ‘molecular handcuff’ 14-3-3 protein after phosphorylation [42,43]. A multitude of other sites, especially serine residues, also influence the regulation. Phosphorylated Ser 338, 471, 494, Thr491 and Tyr341 are stimulatory sites [44,45], while Ser 29, 43, 289, 296, 301 and 642 are inhibitory feedback sites of Erk [42]. In comparison of c-Raf from *Homo sapiens* (Hs-c-Raf), the conserved phosphorylation sites of On-c-Raf included both stimulatory (S348, Y351, S481 and T501) and inhibitory 14-3-3-binding sites (S273 and S631). However, S504 was substituted by alanine in teleost. Moreover, half of the Erk feedback sites (S50, S64 and 303) were well conserved in *O. niloticus*. The conservative property indicated significant functions of

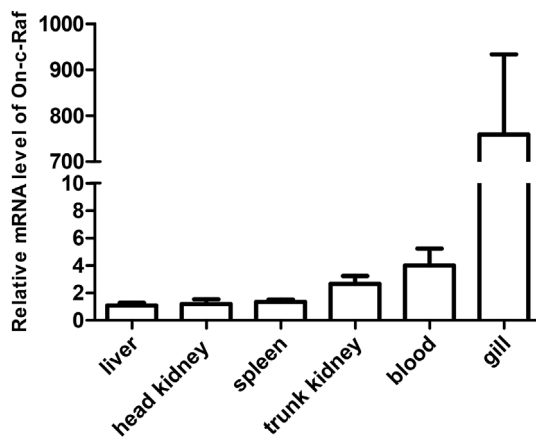


Fig. 4. The mRNA transcripts of On-c-Raf in different tissues detected by RT-PCR. On-c-Raf expression levels of blood, spleen, gill, head-kidney and trunk kidney were normalized to that of liver. The vertical bars represent mean $\pm$ SE (n = 4).

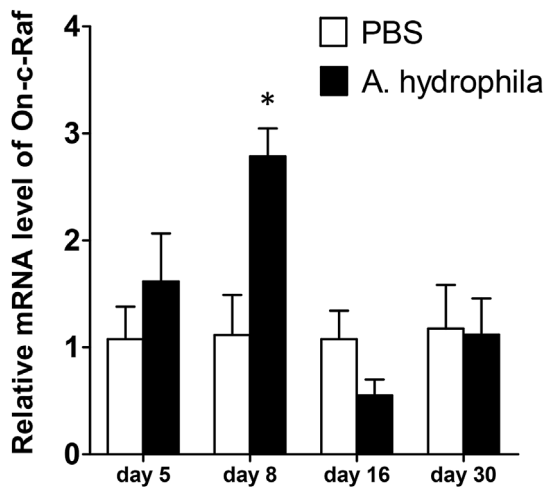


Fig. 5. On-c-Raf responds to *A. hydrophila* stimulation. RT-PCR analysis exhibits the changes of On-c-Raf mRNA level at 5th, 8th, 16th and 30th day post stimulation *in vivo*. The vertical bars represent mean $\pm$ SE (n = 4).

c-Raf in Nile tilapias due to its multiple regulations. When noticing the c-Raf from *Drosophila melanogaster* (Dm-c-Raf), it was less conserved with more substituted stimulatory phosphorylation sites and without matched feedback sites of Erk, which suggested that On-c-Raf might be more regulated compared with that from invertebrates. The prediction of domains and tertiary structures of Rafs showed that the kinase domain was early originated from virus and highly conserved across all of the interested species. The aspartate's rotating into the ATP-binding pocket is critical for the active conformation of c-Raf [46]. Combining the analysis of multiple sequence alignment and homologous modeling, the ATP-binding sequences (Fig. 1) could spatially form an ATP-binding pocket where the active site D478 located nearby (Fig. 2b). The activity of c-Raf can be enhanced through homodimerization with another c-Raf or heterodimerization with B-Raf [47]. Nevertheless, only On-c-Raf and Hs-c-Raf could dimerize in these four Rafs. These hallmarks in sequence and structure, together with well-clustered groups in phylogenetic tree, illustrated that c-Raf was an ancient kinase whose regulation became increasingly precise with species evolving, and the On-c-Raf might play an intermediate role bridging the evolution of c-Rafs.

The tissue expression profile of On-c-Raf contributes to our understanding on its physiology functions. Though the Ras-Raf-Mek-Erk cascade was crucial to multiple cellular functions [7], its studies in aquatic animals were just in infancy. These years, increasing researches

were focused on the downstream molecules of c-Raf and their tissue expressions were detected in invertebrates. For instance, the Erks from *Crassostrea hongkongensis*, *Litopenaeus vannamei* and *Pinctada fucata* were all ubiquitously expressed in different kinds of tissues among which hemocytes, gill, digestive gland and mantle tended to show high expression [48–50]. While the FcMAPKK from *Fenneropenaeus chinensis* was uniquely high expressed in hepatopancreas [51]. However, nothing has been reported about c-Raf in teleost. Here, our study showed that On-c-Raf was widely expressed in various tissues with different levels. As a kind of aquatic animal, Nile tilapias were constantly exposed to water environment that was full of pathogen; meanwhile c-Raf was closely associated with immune responses [7]. Hence the wide expression of On-c-Raf suggested its important role in immune defense of *O. niloticus*. As gill is the organ responsible for gas exchange, it contacts pathogens in water with a high frequency. Therefore, it was speculated that the immunocytes might be locally over-activated in gill and the c-Raf was consequently of high expression. However, further study is still needed to illustrate this tissue expression pattern.

To investigate whether On-c-Raf truly functions in adaptive immunity, its inducible expression during adaptive immune response phase was monitored after challenge of the representative pathogenic bacteria *A. hydrophila*. The members of the MAPK cascade have been proven activated involved in innate immunity after antigen stimulation in invertebrates [48–50], but to our knowledge, there was no report about its functions in adaptive immunity of teleost. Multitudes of experiments indicated that the adaptive immunity dominated pathogen clearance about one week after infection [52–56]. Because *i.p.* injected bacteria caused a systematic but not local infection, in present study we chose the spleen organ that represent systematic immunity for the analysis of c-Raf mRNA expression after infection. Our result showed that during the adaptive immune response, On-c-Raf was significantly upregulated on day 8 post bacterial infection, and the expression came back to original level on day 16 and 30 after infection. These evidences indicated that On-c-Raf might play a pivotal role in the stage of primary response but not memory formation of adaptive immune responses that potentially mediated by lymphocytes.

c-Raf is closely related to lymphocytes activation as the Raf-Mek-Erk is a vital cascade of both TCR and BCR signaling [12,13,21,22]. When this cascade was stimulated, T cells activated and increasingly produced interleukin-2 (IL-2). The constitutive activation of c-Raf could stimulate Erk, while a dominant inhibitory mutant of c-Raf blocked the Erk activation after stimulated by mitogen in a transient transfection cell [57]. In T cell signaling study, PHA and PMA are two widely used reagents. PHA is a specific mitogen to induce T cell activation and proliferation [58], while PMA is an analogue of secondary messenger DAG that directly activates TCR signaling pathway via RasGRP1-Raf-ERK1/2 and PKC θ -NF κ B axis [59,60]. Activation of lymphocytes signaling by PMA is rather drastic and prompt, which is widely used in the phosphorylation study of signaling transduction. However, PHA stimulation is relative mild and slow, which can be used for proliferation study and mRNA level detection. In the present study, to investigate how On-c-Raf participated in adaptive immune responses, we stimulated leukocytes with PHA and found the expression level of On-c-Raf was significantly unregulated after stimulation, indicating that c-Raf regulated lymphocyte activation in Nile tilapias. After stimulation with PMA, phosphorylation of c-Raf occurred within 30 s and lasted for up to 30 min in mammalian T cells [61]. The IL-2 production, which is indispensable for T cell activation and function, could be enhanced in presence of PMA induced c-Raf phosphorylation [62]. In the isolated leukocytes of Nile tilapias, we observed that the phosphorylation of c-Raf was enhanced at 10 min and did not attenuate at 30 min. The rapid increase of p-c-Raf detected by western-blot and immunofluorescent analyses proved that the c-Raf could regulate activation of lymphocytes by increasing phosphorylation. The phosphorylated c-Raf may successively activate Erk, which can be translocated into nucleus and phosphorylates the transcriptional factor that are highly expressed during T cell

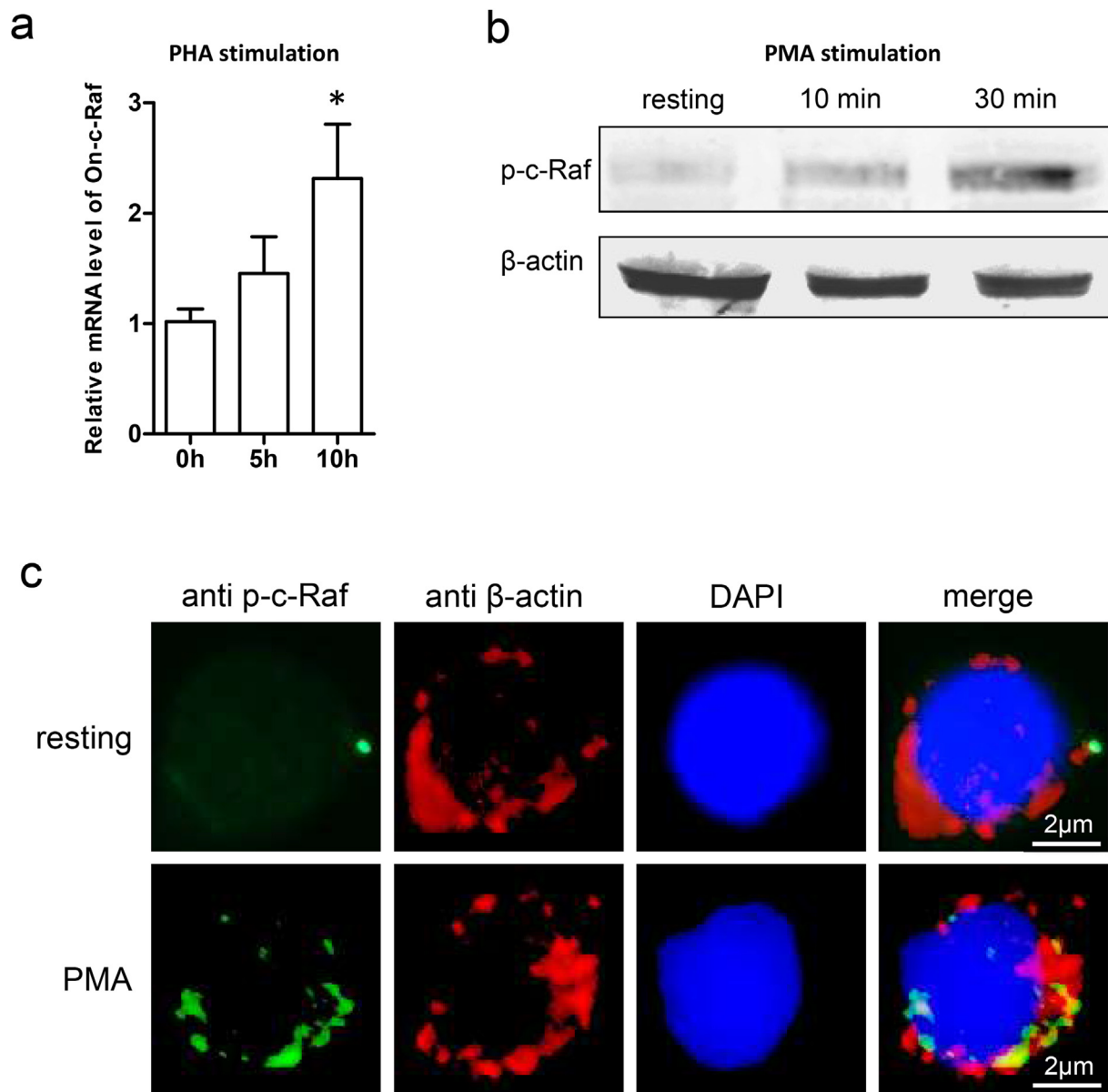


Fig. 6. Activation of On-c-Raf after PHA or PMA stimulation. (a) Upregulation of On-c-Raf after PHA stimulation. RT-PCR assay shows the relative mRNA level of On-c-Raf in leukocytes at 5 h and 10 h post PHA stimulation *in vitro*. The vertical bars represents mean $\pm$ SE (n = 4). (b–c) Phosphorylation of On-c-Raf after PMA stimulation. Immunoblotting analysis shows the phosphorylated On-c-Raf (p-c-Raf) in leukocytes at 10 min and 30 min post stimulation, and β -actin is selected as internal control (b). The leukocytes were rested or collected at 15 min post stimulation for immunofluorescence. Green: Alexa Fluor 488, red: Alexa Fluor 594, blue: DAPI (c). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

and B cell activation [21,22,63]. As a result, the role of c-Raf is speculated to be conserved during lymphocyte activation in Nile tilapia but the specific upstream and downstream mechanisms require further studies.

In summary, we studied an evolutionarily conserved c-Raf in a teleost *O. niloticus*. We proved that the On-c-Raf participated in the adaptive immune responses during anti-bacterial infection and provided the first state that c-Raf was involved in the pathway controlling activation of lymphocytes in teleost. Our research enriched and developed the regulatory mechanism of the adaptive immunity in teleost, providing a new perspective and evidence for the studies on the evolution of adaptive immune system.

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