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# Characterisation of amphioxus protein kinase C-δ/θ reveals a unique proto-V3 domain suggesting an evolutionary mechanism for PKC-θ unique V3

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ABSTRACT

A primitive adaptive immune system has recently been suggested to be present in a basal chordate amphioxus (*Branchiostoma belcheri,* Bb), making it an ideal model for studying the origin of adaptive immune. The novel protein kinase C isoform PKC-θ, but not its closest isoform PKC-δ, plays a critical role for mammalian T-cell activation via translocation to immunological synapse (IS) mediated by a unique PKC-θ V3 domain containing one PxxP motif. To understand the evolution of this unique PKC-θ V3 domain and the primitive adaptive immune system in amphioxus, we comparatively studied the orthologs of PKC-δ and -θ from amphioxus and other species. Phylogenetic analysis showed BbPKC-δ/θ to be the common ancestor of vertebrate PKC-δ and PKC-θ, with a V3 domain containing two PxxP motifs. One motif is conserved in both zebrafish and mammalian PKC-θ but is absent in PKC-δ V3 domain of these species, and has already emerged in drosophila PKC-δ. The other non-conserved motif emerged in BbPKC-δ/θ, and only retained in *Danio rerio* PKC-δ (DrPKC-δ) but lost in mammalian PKC-δ and -θ. Comparative analyses of the sequence and function of BbPKC-δ/θ, DrPKC-δ, DrPKC-θ and *Homo sapiens* PKC-θ (HsPKC-θ) in IS translocation and T-cell receptor (TCR)-induced NF-κB activation revealed that retention of the conserved PxxP motif and loss of the non-conserved PxxP motif in mammalian PKC-θ and loss of both PxxP motifs in mammalian PKC-δ accomplish the unique function of PKC-θ in T cells. Together, this study suggests an evolutionary mechanism for PKC-θ unique V3 and reveals BbPKC-δ/θ is the common ancestor of PKC-δ and -θ with a functional proto-V3 domain, supplying new evidence for the existence of primitive adaptive immune system in amphioxus.

## 1. Introduction

Mammalian protein kinase C-θ (PKC-θ) is a member of the novel, $Ca^{2+}$-independent PKC subfamily (consisting of δ, ε, η and θ subtypes). PKC-θ functions as a critical regulator of T-cell activation by translocating to the T-cell immunological synapse (IS) that forms at the interface between T cells and antigen presenting cells [1,2]. This association facilitates the T-cell receptor (TCR)/coreceptor CD28-induced activation of transcription factors NF-κB, AP-1 and NFAT, and subsequently, interleukin 2 (IL-2) production and T-cell proliferation [3–11]. Human PKC-θ (706 amino acids) consists of a regulatory region (amino acids 1–378) and a catalytic region (amino acids 379–706). The regulatory region displays the domain structure typical of PKC isoforms, containing a C2-like domain structurally similar to the $Ca^{2+}$-binding C2 domain but not binding $Ca^{2+}$ [12], and a phorbol ester– or DAG-binding C1 domain with two tandem cysteine-rich sequences (C1a and C1b), and two variable, or hinge, regions (V1 and V3) [2]. Evolutionarily, members of the novel PKC subfamily can be grouped into the "novel" isoforms PKC-δ and -θ and the distinct "novel" isoforms PKC-ε and -η [13]. PKC-δ, the closest isoform of PKC-θ, shares 62% identity and 75% homology with PKC-θ. However, it does not localise in a stable manner to IS and thus fails to activate sustained TCR signalling [14]. This discrepancy is primarily caused by difference in the V3 domain. The V3 domain of PKC-θ, but not PKC-δ, contains a unique proline-rich (PxxP) motif that is necessary and sufficient for PKC-θ IS recruitment by Lck-dependent association with CD28 [14]. How this specificity was achieved evolutionally remains unknown.

Amphioxus, a chordate invertebrate linking the non-chordate and vertebrate lineages [15] has recently emerged as a novel model system to study the origin of immune system. Functional genomic and experimental studies underscore the value of amphioxus as a model for studying innate immunity [16]. Recently, amphioxus is also suggested to be a novel model for studying the origin of adaptive immunity [16,17]. This is due to the discovery of lymphocyte-like cells in these

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animals as well as the emergence of a proto-MHC region and the identification of histocompatibility-relevant genes and lymphocyte immune signalling-relevant genes [16,18,19]. In addition, the functional identification of *ProtoRAG* [20] and the homologous existence of slp76-signalosome of TCR signalling [21] have been identified in amphioxus. Zebrafish (*Danio rerio*) is a vertebrate fish that possesses an adaptive immune with a complete MHC complex, differentiation of B and T lymphocytes and production of immunoglobulins to fight infection [22,23]. In order to understand the primitive adaptive immune system in amphioxus, a comparative analysis of amphioxus, zebrafish and mammalian PKC-θ and PKC-δ molecule was conducted. These studies also helped decipher the evolutionary origin of the unique PxxP-containing V3 domain of PKC-θ.

In the present study, the cloned amphioxus BbPKC-δ/θ gene was demonstrated to be the common ancestor of PKC-θ and PKC-δ, with a proto-V3 domain containing two PxxP motifs. One motif is conserved in both zebrafish and mammalian PKC-θ but is absent in PKC-δ V3 domain of these species, and has already emerged in *Drosophila melanogaster* PKC-δ (DmPKC-δ). The other non-conserved motif emerged in BbPKC-δ/θ, and only retained in DrPKC-δ but lost in mammalian PKC-δ/θ. Ectopic expression of BbPKC-δ/θ and *Danio rerio* PKC-θ (DrPKC-θ), but not DrPKC-δ, significantly promoted TCR-induced NF-κB activation. Interestingly, BbPKC-δ/θ and DrPKC-δ as well as DrPKC-θ were able to translocate to IS. The V3 domain contributed to the IS recruitment of BbPKC-δ/θ, DrPKC-δ and DrPKC-θ, depending on their PxxP motifs. The PxxP motifs were also essential for the BbPKC-δ/θ and DrPKC-θ promotion of TCR—NF—κB activation. In summary, these findings decipher the evolutionary origin for PKC-θ unique V3, revealing a functional proto-V3 domain in BbPKC-δ/θ.

## 2. Materials and methods

### 2.1. cDNA cloning of BbPKC-δ/θ

A partial sequence of BbPKC-δ/θ was cloned from Chinese amphioxus (*Branchiostoma belcheri*, Qingdao, China) intestinal cDNA by a specific primer pair (Bb-conserved domain-s and Bb-conserved domain-as). This was based on the sequence of *B. floridae* PKC-δ from the JGI database (http://genome.jgi-psf.org). Full-length cDNA was amplified using the 5′ RACE and 3' RACE GeneRACE Kit (Invitrogen, Carlsbad, CA) with primers (3′ race -1st, 3′ race -2nd, 5′ race -1st and 5′ race -2nd) as listed in Table 1.

### 2.2. Plasmids

HsPKC-θ, BbPKC-δ/θ, DrPKC-θ and DrPKC-δ coding regions were amplified from cDNA libraries by their specific primers as listed in Table 1, and then cloned into the expression vector pFlag-CMV-2 (Sigma-Aldrich, E7398). Plasmids encoding the luciferase reporter gene for NF-κB have been previously described [9]. Point mutations were generated by site-directed mutagenesis with a QuikChange Site-Directed Mutagenesis Kit (Stratagene).

### 2.3. Sequence analysis

The amino acid sequences of HsPKC-θ and other PKC-θ and -δ homologs from different species were obtained at the National Center for Biotechnology Information (https://www.ncbi.nlm.nih.gov/). NCBI blast program was used to align the amino acid sequences of different proteins. Prediction of protein domains was accomplished by using an online analysis tool SMART (http://smart.embl-heidelberg.de/). Sequence alignment was conducted based on amino acid sequences using MEGA 5.1 and GENEDOC. A phylogenetic tree was constructed based on the deduced amino acid sequences by the neighbour-joining algorithm with 1000 bootstrap replicates embedded in the MEGA 5.1 program. Disordered regions in proteins were predicted using the Protein Disorder Predictors DISOPRED2 server (http://bioinf.cs.ucl.ac.uk/psipred/?disopred=1) with a prediction threshold set at 5% false positives [24].

### 2.4. Cell culture and transfection

Jurkat E6.1, TAg or Raji cells (American Type Culture Collection) were maintained in complete RPMI-1640 medium supplemented with 10% FBS, penicillin and streptomycin. Cells in a logarithmic growth phase were transfected by nucleofection (Amaxa). In each experiment, cells were transfected with the same total amount of DNA by the addition of appropriate quantities of the empty vector.

### 2.5. Fluorescence microscopy

Fluorescence immunostaining was achieved as described [10]. In brief, Raji B cells.

Were stained for 30 min at 37 °C with CMAC CellTracker Blue (10 μM) in serum-free medium, then washed with serum-free RPMI-1640 medium and incubated for 30 min at 37 °C in the presence or absence of 100 ng/ml SEE (Toxin Technology). Cells were then mixed with Jurkat T cells at the ratio of 1:1, followed by incubation for 20 min at 37 °C. The conjugates were plated on poly-L-lysine-coated slides, followed by incubation for 15 min at room temperature, and then fixed with 4% PFA for 15 min and permeabilised with 0.2% Triton X-100 for 10 min at room temperature. Fixed cells were blocked in 2% BSA for more than 30 min and stained overnight at 4 °C with primary antibodies. Subsequently, the slides were incubated for 1 h at room temperature with Alexa Fluor 488-coupled chicken anti-mouse IgG (A-21200, from Invitrogen). Samples were washed with PBS three times after each step and mounted with a drop of mounting medium. Images were obtained with a ZEISS ImagerZ1 microscopy equipped with 63 × objective lens with laser excitation at 405 and 488 nm. Images were analysed and processed with the AxioVision Rel. 4.8 and Adobe Photoshop CS5 software.

### 2.6. Reporter gene assay

Jurkat T cells were transfected with luciferase reporter plasmids for NF-κB, together with the appropriate expression vectors. A control renilla luciferase reporter vector was used for normalisation. After 40 h, cells were stimulated with 5 μg/ml CD3 monoclonal antibody and 2 μg/ml CD28 monoclonal antibody for 8 h. Cells were harvested and washed with PBS and lysed, and the lysates were assayed for dual luciferase activity (Promega).

### 2.7. Statistical analysis

Statistical analysis was performed with one-way ANOVA followed by Tukey's test analysis of variance. $P$ values of $< 0.05$ were considered statistically significant. GraphPad 5.0 software was used for graphs and statistical analysis.

## 3. Results

### 3.1. Cloning and sequence analysis of BbPKC-δ/θ

By searching the JGI database, the gene named *B. floridae* PKC-δ was shown to have a high homology to HsPKC-θ. Due to the incomplete and inaccurate sequence information of Chinese *B. belcheri* BbPKC-δ/θ, we designed the primers in a conserved region and then cloned a partial sequence of BbPKC-δ/θ. Using the RACE technique, the full-length BbPKC-δ/θ was then cloned. The cloned BbPKC-δ/θ cDNA contains 2454 bp (GenBank accession No. JQ684102), with 150 bp of 5′ untranslated region (UTR), 201 bp of 3′ UTR and 2103 bp of the coding region that encodes 700 amino acid residues (GenBank accession No.

**Table 1**
Primers used in the present study.

| Primer name | Sequence 5′-3′ | Amplification target |
|---|---|---|
| Bb-conserved domain-s | AAAGTGCTGGGCAAGGGCAG | Conserved region of BbPKC-δ/θ |
| Bb-conserved domain-as | TCAGGTGTTCCACAGAAGGT | |
| BbPKC-δ/θ-s | ATGTCTGTTCTGGGAGGACC | target cDNA fragment |
| BbPKC-δ/θ-as | ACTGATAGCAATCTGATTTT | |
| 3′ race-1st | GAGCCACGTTCTACGCCGCTGAAAT | 3′ race |
| 3′ race -2nd | GGATCTCAAGCTGGATAATGTGATG | |
| 5′ race -1st | TGTACAGAGCCACGTTCTACGCCGCTG | 5′ race |
| 5′ race -2nd | CGCCGCTGAAATAGTGTGTGGCTTGC | |
| BbPKC-δ/θ-P330A-s | GAGGTGTCGCCGCCTGCCAGACCACCCAAG | Point mutation |
| BbPKC-δ/θ-P330A-as | CTTGGGTGGTCTGGCAGGCGGCGACACCTC | |
| BbPKC-δ/θ-P333A-s | GCCTCCCAGACCAGCCAAGCCTGGCAAC | |
| BbPKC-δ/θ-P333A-as | GTTGCCAGGCTTGGCTGGTCTGGGAGGC | |
| BbPKC-δ/θ-P368-371A-s | ATGTGGGAAGTGGAGCTGGCCCCCCTGGCCCCACGATCCACCAGC | |
| BbPKC-δ/θ-P368-371A-as | GCTGGTGGATCGTGGGGCCAGGGGGGCCAGCTCCACTTCCCACAT | |
| BbPKC-δ/θ-V3-s | GCCCTGGCACACATCAGC | V3 domain of BbPKC-δ/θ |
| BbPKC-δ/θ-V3-as | CTATTCTTCAGCAAAGTTCC | |
| DrPKC-θ-s | ATGTCTCCGTTTTTGAGGAT | target cDNA fragment |
| DrPKC-θ-as | TCAGGGGCTCTTGAGACGAG | |
| DrPKC-θ-P342A-s | CTTAGCGATTTCCGCCCTTACGCCTG | Point mutation |
| DrPKC-θ-P342A-as | CAGGCGTAAGGGCGGAAATCGCTAAG | |
| DrPKC-θ-P345A-s | GACGCTTAGCGATTTCCGCACTAACCGCTGCACCACCGCTACCAC | |
| DrPKC-θ-P345A-as | GTGGTAGCGGTGGTGCGGCGGTCAGGGCGGAAATCGCTAAGCGTC | |
| DrPKC-θ-V3-s | GCCCTGGCTATGATTGAGAG | V3 domain of DrPKC-θ |
| DrPKC-θ-V3-as | GTCTATGGTGAATTTGTGGT | |
| DrPKC-δ-s | ATGGCCCCTTTCCTGAGAAT | target cDNA fragment |
| DrPKC-δ-as | CTTTGTAATAAGATGCTCCA | |
| DrPKC-δ-P340A-s | CATATATGGGAAGGTTCAAGTGCCCGTCCTCCATCTCGAATCAC | Point mutation |
| DrPKC-δ-P340A-as | GTGATTCGAGATGGAGGACGGGCACTTGAACCTTCCCATATATG | |
| DrPKC-δ-P343A-s | CAAGTCCTCGTCCTGCCTCTCGAATCACCCAC | |
| DrPKC-δ-P343A-as | GTTTGGTGGGTGATTCGAGAGGCGGGCCTGGCACTTGAACCTTCC | |
| DrPKC-δ-V3-s | GCTCTTACTCAAGTCTCAACT | V3 domain of DrPKC-δ |
| DrPKC-δ-V3-as | CTCAGCAGTAATACGTGTTTGG | |
| HsPKC-θ-s | ATGTCGCCATTTCTTCGGAT | target cDNA fragment |
| HsPKC-θ-as | TCAGGATATCAGCCGCTCCATCC | |
| HsPKC-θ-P331A-s | GAAGCAAGGCCGGCCTGTTTACCGACAC | Point mutation |
| HsPKC-θ-P331A-as | GTGTCGGTAAACAGGCCGGCCTTGCTTC | |
| HsPKC-θ-P334A-s | CAAGGCCGCCATGTTTAGCCACACCGGGAAAAAGAG | Point mutation |
| HsPKC-θ-P334A-as | CTTTTTCCCGGTGTGGCCAGGCAGGCCGGCCTTGCTTCATTTTTG | |
| HsPKC-θ-V3-s | GCGCTGGCCATGATTGAG | V3 domain of HsPKC-θ |
| HsPKC-θ-V3-as | CTCAATTTTTAGTTTAATCTGCAG | |

**Table 2**
Protein sequence identity analysis of PKC-θ and -δ homologs.

| | HsPKC-θ | HsPKC-δ |
|---|---|---|
| BbPKC-δ/θ | 55% | 55% |
| DrPKC-θ | 74% | 61% |
| DrPKC-δ | 61% | 76% |

AFH35131.1) (Supplementary Fig. 1). For comparative study, DrPKC-θ (GenBank accession No. NM_001089370.2) and DrPKC-δ (GenBank accession No. NM_214708.1) were cloned from zebrafish. Multiple sequence alignments showed that BbPKC-δ/θ DrPKC-θ and DrPKC-δ shared 55%, 74% and 61% identity, respectively, with HsPKC-θ and 55%, 61% and 76% identity, respectively, with HsPKC-δ (Table 2). These results showed the same degree of homology (55%) between BbPKC-δ/θ and HsPKC-θ and between BbPKC-δ/θ and HsPKC-δ. Protein domains prediction showed that similar to HsPKC-θ and HsPKC-δ, BbPKC-δ/θ, DrPKC-θ and -δ have an N-terminal regulatory region with a C2-like domain, a C1 domain and a C-terminal catalytic region (Fig. 1).

Using MEGA 5.1 software, a neighbour-joining phylogenetic tree was conducted of PKC-θ and PKC-δ orthologs from drosophila, sea urchin, starfish, amphioxus, zebrafish, coelacanth and various mammalian species. Phylogenetic analysis showed that BbPKC-δ/θ is the common ancestor of vertebrate PKC-θ and PKC-δ that quite possibly diverged into vertebrate PKC-θ and PKC-δ over the course of evolution. The evolutionary position of BbPKC-δ/θ is in accordance with the transition status of amphioxus in the evolution of chordates (Fig. 2).

### 3.2. Functional characterisation of BbPKC-δ/θ, DrPKC-θ and DrPKC-δ in TCR signalling

Next, it was investigated whether BbPKC-δ/θ, DrPKC-θ or DrPKC-δ could promote TCR-induced NF-κB activity in T cells. Exogenous expression of HsPKC-θ promoted CD3/CD28-induced NF-κB activity, as expected. Interestingly, exogenous expression of both BbPKC-δ/θ and DrPKC-θ significantly increased CD3/CD28-induced NF-κB activity, where DrPKC-θ was stronger than BbPKC-δ/θ in the activation of NF-κB. However, DrPKC-δ slightly promoted NF-κB activation compared with the vector control in T cells upon stimulation (Fig. 3A). These results indicated that like HsPKC-θ and -δ, zebrafish DrPKC-θ and -δ already have entirely diverged functions in TCR-induced NF-κB activation. Nevertheless, BbPKC-δ/θ possesses certain ability of HsPKC-θ in TCR-induced NF-κB activation, in alignment with the results of the phylogenetic analysis indicating that BbPKC-δ/θ is the common ancestor of PKC-θ and -δ.

Unique IS translocation of HsPKC-θ is essential for its role in TCR-induced NF-κB activation. Thus, we checked whether BbPKC-δ/θ,

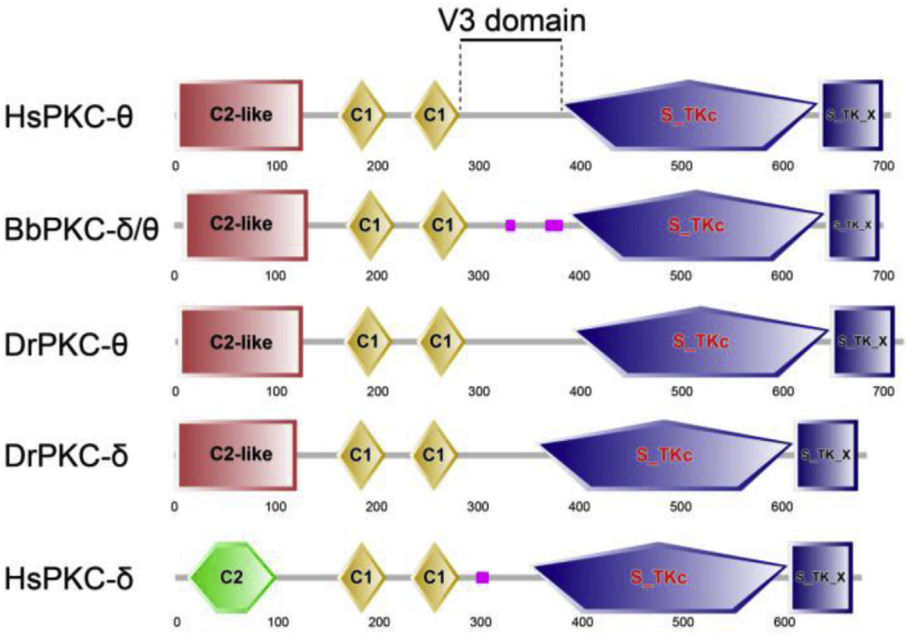


**Fig. 1. Domain topology of PKC-θ and -δ orthologs.** The protein domains were predicted by online analysis tool SMART (http://smart.embl-heidelberg.de/). Pink boxes indicate a low-complexity region. The V3 domain of HsPKC-θ was also highlighted. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

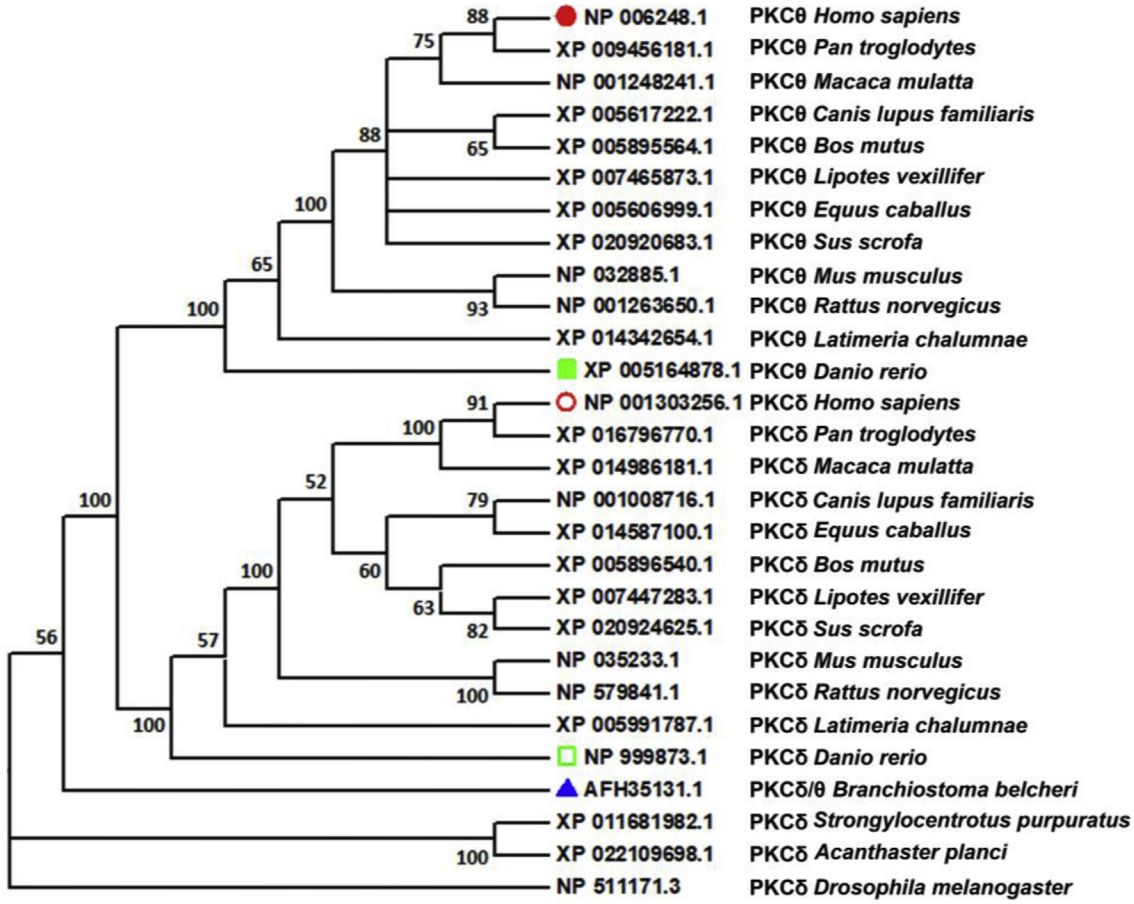


**Fig. 2. Phylogenetic tree of PKC-θ and -δ homologs.** A neighbour-joining phylogenetic tree was constructed on the basis of 28 different PKC-θ or PKC-δ sequences from GenBank, utilising the sequence analysis tool MEGA 5.1. Numbers at tree nodes indicate the bootstrap values from 1000 replicates. HsPKC-θ, DrPKC-θ, DrPKC-δ and BbPKC-δ/θ were highlighted with different shapes and colours. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

DrPKC-θ or DrPKC-δcould translocate to IS following antigen stimulation. BbPKC-δ/θ, DrPKC-θ, DrPKC-δ and the control HsPKC-θ expression plasmids were transfected into Jurkat T cells. At 48 h post-transfection, cells were stimulated with SEE-pulsed Raji B cells for 20 min and fixed for fluorescence analysis. As expected, HsPKC-θ translocated to IS after stimulation. Interestingly, not only DrPKC-θ but

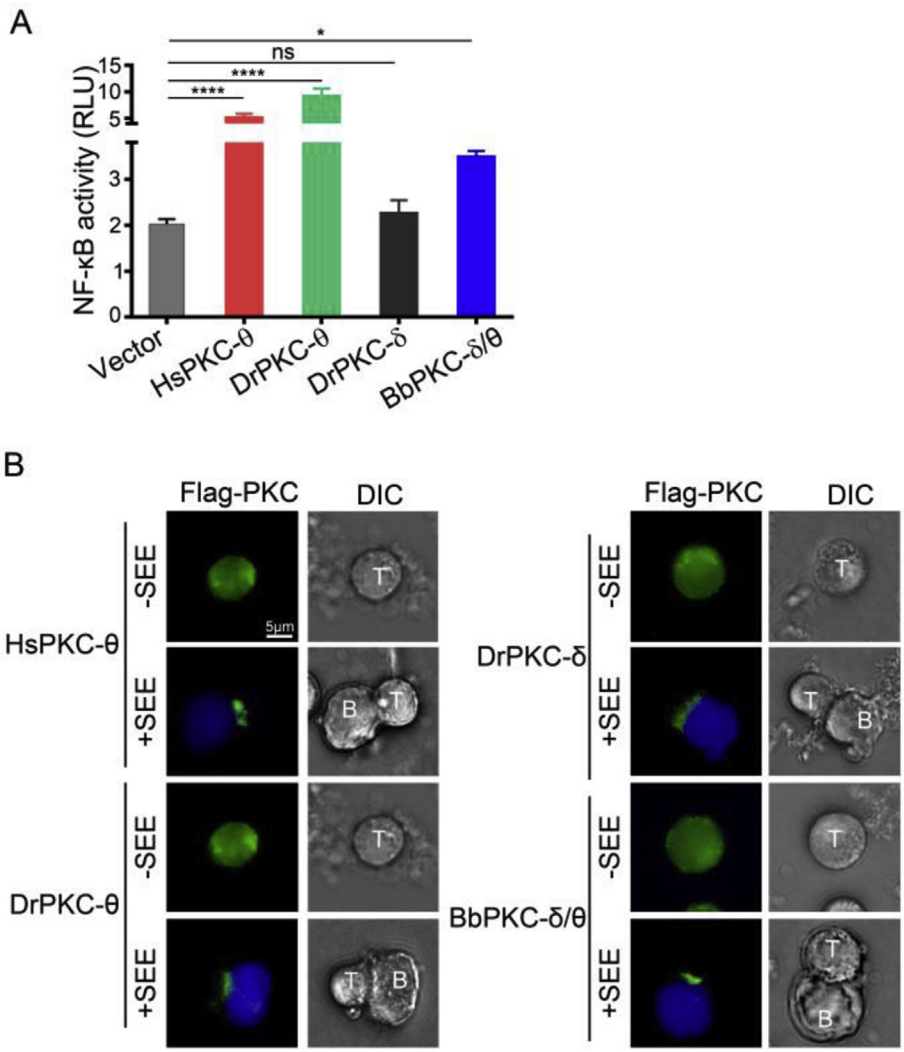


**Fig. 3. PKC-θ and -δ orthologs-mediated NF-κB activation and IS translocation in Jurkat T cells.** (A) Normalised Luc activity in Jurkat T cells co-transfected with the empty Flag-CMV-2 vector or the indicated PKC-θ and–δ constructs together with NF-κB-Luc and Renilla reporter plasmids. Cells were co-stimulated with anti-CD3 and anti-CD28 for 8 h. Data are expressed as fold increase in NF-κB activity, as compared to vector group (unstimulated). ns: not significance, *$p < 0.05$, ****$p < 0.0001$ (one-way ANOVA followed by Tukey's test). RLU, relative light units. Results are shown as mean ± s.e.m. (from two (HsPKC-θ and DrPKC-θ, δ) or three (BbPKC-δ/θ) independent experiments). (B) Image analysis of the intracellular localisation of the PKC-θ and–δ orthologs (green) in Jurkat T cells transfected with the indicated plasmids respectively, and stimulated for 20 min with SEE-pulsed CMAC-labelled Raji cells (blue). Scale bar, 5 μm. Data are representative of three independent experiments. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

also BbPKC-δ/θ and DrPKC-δ could translocate to IS (Fig. 3B). These results suggested that similar to HsPKC-θ, BbPKC-δ/θ, DrPKC-θ and DrPKC-δ likely contain the motif needed for IS translocation, although DrPKC-δ could not promote NF-κB activation in T cells.

### 3.3. Identification of a proto-V3 domain in BbPKC-δ/θ

The unique IS localisation of HsPKC-θ requires the V3 domain, which contains a PxxP motif (ARPPCLPTP, proline residues underlined) that does not exist in the V3 domain in HsPKC-δ. The PxxP motif mediates HsPKC-θ binding to the Lck SH3 domain and, in turn, facilitates IS translocation [14]. Thus, the V3 domains of BbPKC-δ/θ, DrPKC-θ and DrPKC-δ were compared with HsPKC-θ. NCBI BLAST program analysis showed that the V3 domain has a substantial divergence between HsPKC-δ and HsPKC-θ, as previously reported [14] (see Fig. 4A), yet with very little divergence between BbPKC-δ/θ and HsPKC-θ or between DrPKC-θ and HsPKC-θ. However, there was some divergence between DrPKC-δ and HsPKC-θ (Fig. 4A). These results indicated that compared with the V3 domain of HsPKC-δ, those of DrPKC-θ, BbPKC-δ/θ and DrPKC-δ (in decreasing order) are much closer to HsPKC-θ in sequence. Next, the evolutionary conservation of the PxxP motif of HsPKC-θ V3 domain was analysed. Sequence alignment showed that the PxxP motif (ARPPCLPTP), especially the two internal proline residues, is phylogenetically conserved in the V3 domains of mammalian and zebrafish PKC-θ orthologs (Fig. 4B). By manually editing the sequence alignment of the V3 domains of BbPKC-δ/θ and DmPKC-δ with PKC-θ orthologs, the PxxP motif of HsPKC-θ was also found conserved in *Drosophila* (RDSPSTPPS) and amphioxus (SPPPRPPKP). Interestingly, the BbPKC-δ/θ V3 domain has a non-conserved PxxP motif (LPPLPPR) right after the conserved PxxP motif (Fig. 4B). Surprisingly, this non-conserved PxxP motif does not exist in *Drosophila* PKC-δ, zebrafish, coelacanth or in the listed mammalian PKC-θ orthologs (Fig. 4B).

The sequences of PKC-δ from amphioxus, *Drosophila*, zebrafish, coelacanth and mammalian species were further aligned. By manually editing the alignment of the V3 domain of DrPKC-δ with BbPKC-δ/θ, the non-conserved PxxP motif was found in the V3 domain of zebrafish PKC-δ but not in that of *Drosophila*, coelacanth and mammalian species (Fig. 4C).

In summary, BbPKC-δ/θ has a proto-V3 domain that contains conserved and non-conserved PxxP motifs. Mammalian PKC-δ V3 domain does not contain the PxxP motif, while DrPKC-δ only contains the non-conserved domain, and the mammalian PKC-θ only retains the conventional one.

### 3.4. Contribution of the V3 domain and PxxP motif to IS translocation of BbPKC-δ/θ and DrPKC-δ

Since BbPKC-δ/θ, DrPKC-δ and DrPKC-θ have PxxP motifs in the V3 domain, the IS translocation capacity was tested. Fluorescence analysis showed that like the V3 domain of HsPKC-θ, the V3 domains of BbPKC-δ/θ, DrPKC-δ and DrPKC-θ enabled translocation to IS after stimulation

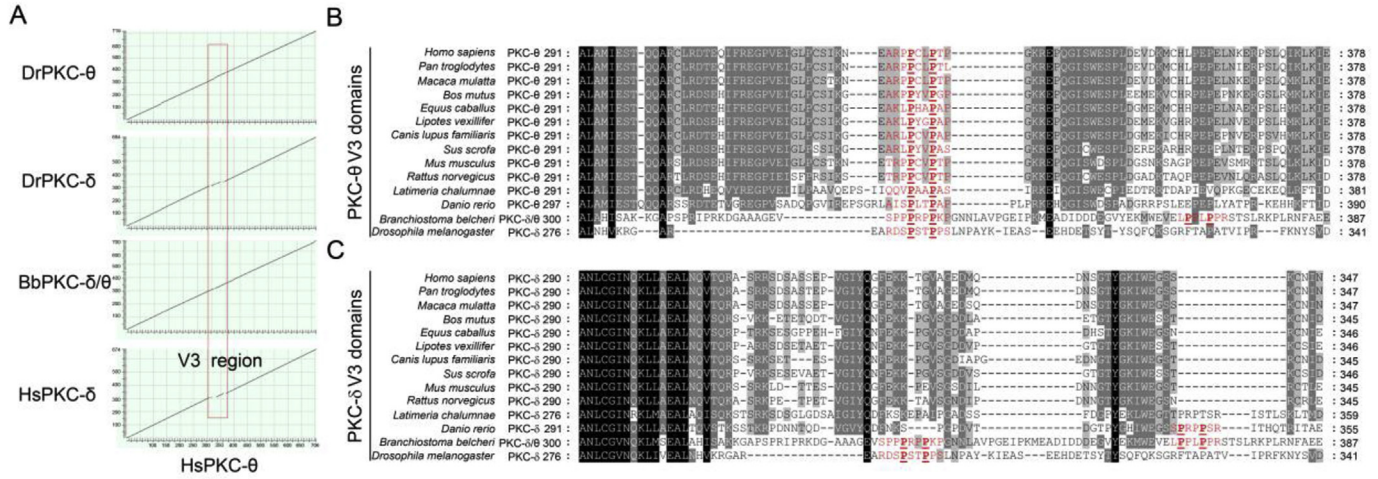


**Fig. 4. Analysis of the V3 domain of PKC-θ and -δ orthologs.** (A) Alignment of HsPKC-θ (NP_006248) and HsPKC-δ (NP_006245), DrPKC-θ (XP_005164878.1), PKC-δ (NP_999873.1), BbPKC-δ/θ (AFH35131.1) showing the divergence of the V3 (hinge) region. Sequences were aligned using the NCBI BLAST program. Alignment of the V3 domains of the indicated PKC-θ homologs (B) and PKC-δ orthologs (C), the PxxP motifs were marked in red and the two main proline amino acids were marked in bold font and underlined. The species analysed in (B) and (C) were the same as in Fig. 2. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

with SEE-pulsed Raji B cells (Fig. 5A–D). To test whether the PxxP motif was required for V3 domain IS localisation, the two internal proline residues (PxxP) were replaced with alanine (AxxA) in the PxxP motif of BbPKC-δ/θ (conserved PxxP: 330–333 aa or non-conserved PxxP: 368–371 aa), DrPKC-θ (conserved PxxP: 342–345 aa) and DrPKC-δ (non-conserved PxxP: 340–343 aa). The HsPKC-θ-V3-PP/AA mutant (331-334aa) served as the control. As previously reported [14], we observed that the HsPKC-θ-V3-PP (331–334)/AA mutant could not translocate to IS (Fig. 5A). Interestingly, both BbPKC-δ/θ-V3-PP (330, 333)/AA and −PP (368, 371)/AA mutants as well as the DrPKC-θ-V3-PP (342, 345)/AA and DrPKC-δ-V3-PP (340, 343)/AA mutants failed to localise to IS and remained largely cytosolic (Fig. 5B–D). These results indicated that the two PxxP motifs in the proto-V3 domain of BbPKC-δ/θ both were required for IS recruitment. The conserved PxxP motif in DrPKC-θ or the non-conserved PxxP motif in DrPKC-δ were required for IS recruitment.

### *3.5. BbPKC-δ/θ and DrPKC-θ PxxP motif mutants failed to promote NF-κB activation*

It was further analysed whether the PxxP motif mutation in BbPKC-δ/θ and DrPKC-θ V3 domains influenced TCR-induced NF-κB activation. Corresponding to the deficiency in IS translocation, DrPKC-θ-V3-PP (342, 345)/AA, BbPKC-δ/θ-V3-PP (330, 333)/AA and −PP (368, 371)/AA mutants failed to promote NF-κB activation, similar to HsPKC-θ-V3-PP (331–334)/AA, compared with the wild-type counterparts in T cells upon stimulation (Fig. 6). Taken together, these results indicated that the two PxxP motifs in the proto-V3 domain of BbPKC-δ/θ and the conserved PxxP motif in DrPKC-θ are essential for promoting NF-κB activation in T cells upon stimulation.

## 4. Discussion

In this study, BbPKC-δ/θ was shown to be the common ancestor of PKC-δ and PKC-θ in sequence and function. The data presented here revealed that BbPKC-δ/θ has a proto-V3 domain with two PxxP motifs, a conserved motif and a non-conserved motif, both contributing to IS translocation and NF-κB activation. These data imply that amphioxus PKC-δ/θ may function in amphioxus immunity, providing new evidence for the primitive adaptive immune system present in lower chordates. This study also suggests that the retention of the conserved PxxP motif and the loss of the non-conserved motif in mammalian PKC-θ as well as the loss of both PxxP motifs in mammalian PKC-δ have evolved the mammalian PKC-θ as the unique PKC isoform responsible for IS translocation.

Proline-rich PxxP motifs are known to bind Src homology 3 (SH3) [25]. Mammalian PKC-θ, but not PKC-δ, has a conserved PxxP motif in its V3 domain that interacts with the SH3 domain of Lck. This leads to the association of PKC-θ with TCR-coreceptor CD28 that binds to the Lck SH2 domain, resulting in the unique IS translocation and function of PKC-θ in T cells [14]. Interestingly, the amphioxus PKC-δ V3 domain not only possesses this conserved PxxP motif but also contains a non-conserved PxxP motif (LPPLPPR). This construct belongs to a typical class II PxxP motif (xPxXPx+, where X is generally a hydrophobic residue, x is any residue, + is a positively charged residue), which could interact with the SH3 domain of Src kinase and adaptor protein such as GADs [26]. These two PxxP motifs are both required for BbPKC-δ/θ IS translocation, indicating the regulation of BbPKC-δ/θ is different from HsPKC-δ. This also implies that both the amphioxus ortholog of Lck and an unknown SH3-containing adaptor, or tyrosine kinase, may be required for activation of BbPKC-δ/θ.

After BbPKC-δ/θ diverged into PKC-δ and PKC-θ, PKC-θ only retains the conserved PxxP motif. PKC-δ lost both PxxP motifs, with the exception of zebrafish PKC-δ, which still retains the non-conserved PxxP motif contributing to IS translocation of the DrPKC-δ V3 domain. It should be noted that *Drosophila* PKC-δ only has the conserved PxxP motif. Short linear motifs are continuously created and destroyed by mutations in rapidly evolving disordered regions, creating a dynamic supply of new interactions that may have advantageous phenotypic novelty [27]. Consistent with this theory, the V3 domain of PKC-θ or -δ was a disordered region predicted by the protein disorder predictors DISOPRED2 server (Supplementary Fig. 2). Therefore, the absence of the non-conserved PxxP motif in DmPKC-δ, and its emergence in BbPKC-δ/θ but loss in PKC-θ and mammalian PKC-δ, may have resulted from random mutations under positive or negative selection. Relative to the importance of the conserved PxxP motif of BbPKC-δ/θ and PKC-θ in NF-κB activation, DrPKC-δ could not mediate TCR-induced NF-κB activation even though the non-conserved PxxP motif mediates DrPKC-δ V3 domain IS translocation. These data strengthen the importance of the conserved PxxP motif in the V3 domain of PKC-θ in NF-κB activation.

Although amphioxus does not have T cells, the presence of lymphocyte-like cells has been reported [28]. The discovery of a proto-MHC region, histocompatibility-relevant genes, and lymphocyte

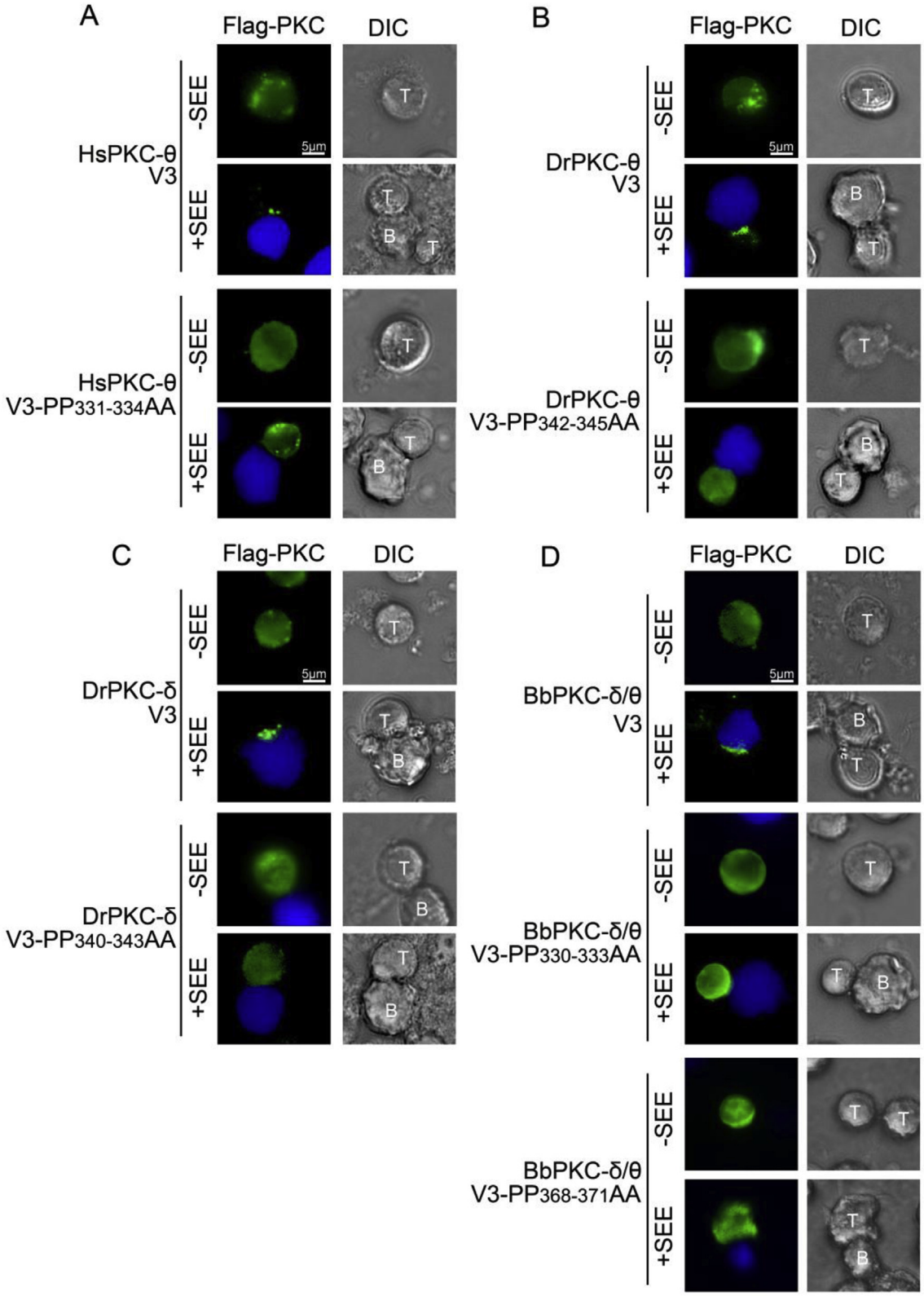


**Fig. 5. IS Translocation analysis of the V3 domains and PP/AA mutants of PKC-θ and PKC-δ orthologs.** Image analysis of the intracellular localisation of the V3 domain and PP/AA mutants (green) in Jurkat T cells transfected with the HsPKC-θ V3 domain (A), DrPKC-θ V3 domain (B), DrPKC-δ V3 domain (C), and BbPKC-δ/θ V3 domain (D) followed by stimulation for 20 min with SEE-pulsed CMAC-labelled Raji cells (blue). Scale bar, 5 μm. Data are representative of three independent experiments.

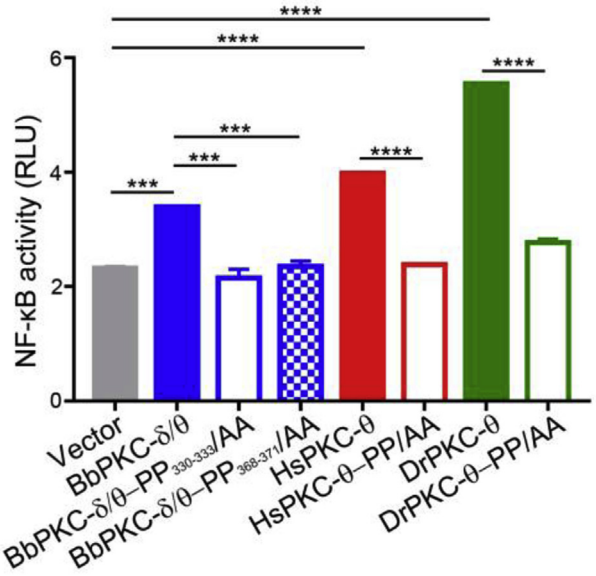


**Fig. 6. Mutation of PxxP motifs impaired PKC-θ and -δ orthologs-mediated NF-κB activation in Jurkat T cells.** Normalised Luc activity in Jurkat T cells co-transfected with empty Flag-CMV-2 vector or the indicated PKC-θ and -δ constructs together with NF-κB-Luc and Renilla reporter plasmids. Cells were co-stimulated with anti-CD3 and anti-CD28 for 8 h. Data are expressed as fold increase in NF-κB activity, as compared to vector group (unstimulated). ***$p < 0.001$, ****$p < 0.0001$ (one-way ANOVA followed by Tukey's test). RLU, relative light units. Results are shown as mean ± s.e.m.

immune signalling-relevant genes [16,18,19], as well as the functional identification of a *ProtoRAG* and BbSlp76 signalosome [20,21], indicates a functional primitive adaptive immune response in amphioxus. BbPKC-δ/θ translocation to IS and activation of NF-κB in T cells further suggests that the lymphocyte-like cells might recognise antigen through the assembly of synapse-like structure in amphioxus, providing a new direction for future characterisation of the primitive adaptive immunology in amphioxus.

In conclusion, our findings revealed that BbPKC-δ/θ is similar to PKC-θ in sequence and function, suggesting that the uniqueness of PKC-θ in T-cell activation may be generated by the retention or loss of PxxP motifs. These results suggest that amphioxus is a valuable model for studying the origin of adaptive immunity.

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## Appendix A. Supplementary data

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## References

[1] C.R. Monks, H. Kupfer, I. Tamir, A. Barlow, A. Kupfer, Selective modulation of protein kinase C-theta during T-cell activation, Nature 385 (1997) 83–86.
[2] A. Altman, K.F. Kong, Protein kinase C enzymes in the hematopoietic and immune systems, Annu. Rev. Immunol. 34 (2016) 511–538.
[3] E.Y. Zhang, K.F. Kong, A. Altman, The yin and yang of protein kinase C-theta (PKCtheta): a novel drug target for selective immunosuppression, Adv. Pharmacol. 66 (2013) 267–312.
[4] K.Q. Zuo, D. Li, B. Pulli, F. Yu, H.D. Cai, X.Y. Yuan, et al., Short-hairpin RNA-mediated Heat shock protein 90 gene silencing inhibits human breast cancer cell growth in vitro and in vivo, Biochem Bioph Res Co 421 (2012) 396–402.
[5] N. Coudronniere, M. Villalba, N. Englund, A. Altman, NF-kappa B activation induced by T cell receptor/CD28 costimulation is mediated by protein kinase C-theta, Proc. Natl. Acad. Sci. U. S. A. 97 (2000) 3394–3399.
[6] X. Lin, A. O'Mahony, Y. Mu, R. Geleziunas, W.C. Greene, Protein kinase C-theta participates in NF-kappaB activation induced by CD3-CD28 costimulation through selective activation of IkappaB kinase beta, Mol. Cell Biol. 20 (2000) 2933–2940.
[7] C. Pfeifhofer, K. Kofler, T. Gruber, N.G. Tabrizi, C. Lutz, K. Maly, et al., Protein kinase C theta affects Ca2+ mobilization and NFAT cell activation in primary mouse T cells, J. Exp. Med. 197 (2003) 1525–1535.
[8] Z. Sun, C.W. Arendt, W. Ellmeier, E.M. Schaeffer, M.J. Sunshine, L. Gandhi, et al., PKC-theta is required for TCR-induced NF-kappaB activation in mature but not immature T lymphocytes, Nature 404 (2000) 402–407.
[9] Y.Q. Li, C.E. Sedwick, J.R. Hu, A. Altman, Role for protein kinase C theta (PKC theta) in TCR/CD28-mediated signaling through the canonical but not the non-canonical pathway for NF-kappa B activation, J. Biol. Chem. 280 (2005) 1217–1223.
[10] X.D. Wang, Y. Gong, Z.L. Chen, B.N. Gong, J.J. Xie, C.Q. Zhong, et al., TCR-induced sumoylation of the kinase PKC-theta controls T cell synapse organization and T cell activation, Nat. Immunol. 16 (2015) 1195–1203.
[11] Y. Li, J. Hu, R. Vita, B. Sun, H. Tabata, A. Altman, SPAK kinase is a substrate and target of PKCtheta in T-cell receptor-induced AP-1 activation pathway, EMBO J. 23 (2004) 1112–1122.
[12] H. Pappa, J. Murray-Rust, L.V. Dekker, P.J. Parker, N.Q. McDonald, Crystal structure of the C2 domain from protein kinase C-delta, Structure 6 (1998) 885–894.
[13] W.S. Sossin, Isoform specificity of protein kinase Cs in synaptic plasticity, Learn. Mem. 14 (2007) 236–246.
[14] K.F. Kong, T. Yokosuka, A.J. Canonigo-Balancio, N. Isakov, T. Saito, A. Altman, A motif in the V3 domain of the kinase PKC-theta determines its localization in the immunological synapse and functions in T cells via association with CD28, Nat. Immunol. 12 (2011) 1105–1112.
[15] N.H. Putnam, T. Butts, D.E. Ferrier, R.F. Furlong, U. Hellsten, T. Kawashima, et al., The amphioxus genome and the evolution of the chordate karyotype, Nature 453 (2008) 1064–1071.
[16] S.C. Yuan, J. Ruan, S.F. Huang, S.W. Chen, A.L. Xu, Amphioxus as a model for investigating evolution of the vertebrate immune system, Dev. Comp. Immunol. 48 (2015) 297–305.
[17] S.C. Zhang, Y.J. Liang, G.D. Ji, Z.M. Zhuang, Protochordate amphioxus is an emerging model organism for comparative immunology, Prog Nat Sci-Mater. 19 (2009) 923–929.
[18] L. Abi-Rached, A. Gilles, T. Shiina, P. Pontarotti, H. Inoko, Evidence of en bloc duplication in vertebrate genomes, Nat. Genet. 31 (2002) 100–105.
[19] Z.H. Liu, L. Li, H.Y. Li, S.C. Zhang, G.D. Ji, Y.L. Sun, EST analysis of the immune-relevant genes in Chinese amphioxus challenged with lipopolysaccharide, Fish Shellfish Immunol. 26 (2009) 843–849.
[20] S. Huang, X. Tao, S. Yuan, Y. Zhang, P. Li, H.A. Beilinson, et al., Discovery of an active RAG transposon illuminates the origins of V(D)J recombination, Cell 166 (2016) 102–114.
[21] X. Qu, X. Lan, C. Deng, J. Zhou, J. Du, S. Huang, et al., Molecular mechanisms underlying the evolution of the slp76 signalosome, Sci. Rep. 7 (2017) 1509.
[22] S.A. Renshaw, N.S. Trede, A model 450 million years in the making: zebrafish and vertebrate immunity, Dis Model Mech 5 (2012) 38–47.
[23] L.Y. Zhu, L. Nie, G. Zhu, L.X. Xiang, J.Z. Shao, Advances in research of fish immune-relevant genes: a comparative overview of innate and adaptive immunity in teleosts, Dev. Comp. Immunol. 39 (2013) 39–62.
[24] J.J. Ward, J.S. Sodhi, L.J. McGuffin, B.F. Buxton, D.T. Jones, Prediction and functional analysis of native disorder in proteins from the three kingdoms of life, J. Mol. Biol. 337 (2004) 635–645.
[25] B.K. Kay, M.P. Williamson, P. Sudol, The importance of being proline: the interaction of proline-rich motifs in signaling proteins with their cognate domains, Faseb. J. 14 (2000) 231–241.
[26] K. Saksela, P. Permi, SH3 domain ligand binding: what's the consensus and where's the specificity? FEBS Lett. 586 (2012) 2609–2614.
[27] N.E. Davey, M.S. Cyert, A.M. Moses, Short linear motifs - ex nihilo evolution of protein regulation, Cell Commun. Signal. 13 (2015) 43.
[28] G. Huang, X. Xie, Y. Han, L. Fan, J. Chen, C. Mou, et al., The identification of lymphocyte-like cells and lymphoid-related genes in amphioxus indicates the twilight for the emergence of adaptive immune system, PloS One 2 (2007) e206.