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Molecular Cloning, Expression, and Enzymatic Analysis of Protein kinase C β I and β II from Inshore hagfish (*Eptatretus burgeri*)

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먹장어 유래의 Protein kinase C βI 과 βII의 분자생물학적 클로닝, 발현, 효소학적 분석

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Abstract

Inshore hagfish (*Eptatretus burgeri*) belongs to chordate and cyclostomata, so it is considered to be an important organism for the study of embryology and biological evolution. Protein kinase C (PKC) performs a wide range of biological functions regarding proliferation, apoptosis, differentiation, motility, and inflammation with cellular signal transduction. In this study, PKC beta isoforms, a member of the conventional class, were cloned. As a result, *EbPKC \beta I* and *EbPKC \beta II* showed the same sequence in conserved regions (C1, C2, C3, and C4 domain), but not in the C-terminal called the V5 domain. The ORFs of *EbPKC \beta I* and *EbPKC \beta II* were 2,007 bp and 2,004 bp, respectively. In the analysis of tissue specific expression patterns by *qPCR*, *EbPKC \beta I* was remarkably highly expressed in the root of the tongue and the spinal cord, while EbPKC βII was highly expressed in the gill, liver, and gut. The *EbPKC* βI and *EbPKC \beta II* expressed in *E. coli* revealed PKC activity according to both qualitative analysis and quantitative analysis.

Key word : PKC & I, PKC & II, Cloning, Expression, Enzymatic analysis, Hagfish

I. Introduction

Inshore hagfish (*Eptatretus burgeri*) lives 10~270m under the seabed near Jeju Island in South Korea, South Sea, and the vicinity of Pacific Northwest. It belongs to chordate and cyclostomata, the lowest groups among vertebrates. Hagfish has atrophied eyes, tentacles, a well-developed tongue, mucous glands, and only one caudal fin, except for

other fins, such as dorsal fin. Studying fish that are considered to be the early stages of vertebrates could help elucidate the embryogenic system and biological evolution (Das, 2012). Hagfish is considered a commercially viable species because of its edibility and utility in leather industry. However, it has been reported that the hagfish population is declining due to overfishing in the littoral sea. In order to maintain the hagfish population, it is

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necessary to study their spawning ecology, and it is thought that molecular biological research data on inshore hagfish must be evaluated.

Protein kinase C (PKC) is a family of enzymes involved in a wide range of biological functions. PKCs have 11 subtypes, which have been categorized into conventional (α , β I, β II and γ), novel (δ , ε , θ and η) and atypical (ζ and ι / λ) classes (Newton, 1995). The regulatory domains of conventional PKC (cPKC) isoforms contain a C1 domain that functions as a DAG-/PMA-binding motif (Baneyx and Mujacic, 2004). cPKC regulatory domains also contain a C2 domain that binds anionic phospholipids in a calcium-dependent manner (Blakey et al., 2005). Novel PKCs (nPKCs) also have twin C1 domains as well as a C2 domain. Importantly, nPKC C2 domains lack the critical calcium-coordinating acidic residues. Atypical PKCs (aPKCs) lack а calcium-sensitive C2 domain; they contain an atypical C1 domain that binds PIP3 or ceramide instead. PKC *β* I and βΠ activated hv diacylglycerol (DAG) and calcium ions perform a range of biological functions regarding wide proliferation, apoptosis, differentiation, motility, and inflammation with cellular signal transduction (Kawakami et al., 2002; Newton, 1995; Wightman and Raetz, 1984).

PKC β I and β II have four identical conserved (C1-C4) regions, but they have about 50 different amino acids in the C-terminal called V5 domains. V5 domains have 50-70 amino acid sequences; COOH-terminal to the catalytic core of the enzyme (C3 and C4 domains) that contain the highly conserved turn and hydrophobic phosphorylation motifs as well as an additional 7-21 residues at the extreme COOH terminus (beyond the hydrophobic motif), that are highly variable in both length and sequence (Schreiber et al., 2001). These regions were generally ignored in early studies exploring the structural determinants of PKC isoform function (Steinberg, 2008). However, V5 domains have recently emerged as structures that impart important determinants of PKC isoform-specific targeting and function, suggesting that V5 domains may represent novel targets for pharmaceuticals designed to regulate PKC isoform-specific signaling in cells (Bobeszko et al., 2004; Cole and Igumenova, 2015; Newton, 1995).

PKC β I and PKC β II are expressed in a tissue-specific and developmentally regulated manner (Gopal and Kumar, 2013). RACK1 anchors PKC β II (but not PKC β I) to the perinuclear region; a PKC β I selective RACK protein is yet to be identified (Ono et al., 1989). While RACK1 binding sites were initially mapped to the PKC β C2 domain, C2 domain RACK1 binding sites (which are common to PKC β I and PKC β II) do not explain the in vivo specificity of RACK1 for PKC β II (Ohno et al., 1968). Rather. the RACK1-binding specificity has been attributed to protein-protein interaction motifs in the V5 domain that are unique to PKC β II (62 1 ACGRNAE⁶²⁷, 6 4 5QEVIRN⁶⁵⁰, and 660SFVNSEFLKPEVKS⁶⁷³) and not found in PKC β I (Donoghue and Purnell, 2005).

Thus, we carried out the molecular research on PKC β I with wide physiology function in inshore hagfish.

II. Materials and Methods

1. cDNA cloning for the complete coding sequence of *Eptatretus burgeri* PKC β I and β II

The total RNA was obtained from 11 tissues (brain, tentacle, gill, root of the tongue, spinal cord, heart, liver, gut, muscle, skin, and mucous gland) of inshore hagfish using GeneAll® Hybrid-R[™] Total RNA (GeneAll Biotechnology Co., Ltd., Korea) as described by previous manuscripts. The RNA was reverse-transcribed by oligo (dT)18 using the PrimeScript[™] 1st strand cDNA Synthesis Kit (TaKaRa, Korea) and random hexamer primers. Several PKC β nucleotide sequences were collected from other species. Degenerated primers were designed around the highly conserved parts of the sequences using BioEdit Sequence Alignment Editor version 5.0.9 (sense primers, DgPKC β -F1 and DgPKC β -F2; antisense primer, DgPKC β -R1 and DgPKC β -R2,

<Table 1> Oligonucleotide primers used in PCR amplification of PKC \$\varsis I\$ and \$\varsis II\$ genes of *E. burgeri* (F, Forward; R, Reverse)

Primer name	5'-3' sequence	Information
DgPKCβ-F1	GGC AAG GTS CTG YTG GCA GAR GAG	
DgPKCβ-F2	CAC TTC CTB ACV TCG CTC TAC TGC GCC	
DgPKCβ-R1	CCA STT CTG GGY TTG AAV GGH GGC	
DgPKCβ-R2	GCM GGG TKC TTB GTS AGG AGB CC	Primers for
3'GSP-EbPKCβ-F1	GTG GAA CTC CAG ACT ATA TCG CTC C	cDNA library screening
3'GSP-EbPKCβ-F2	GGC TGG AGA GCC GCC ATT TGA TGG TG	
5'GSP-EbPKCβ-R1	GCG CCT GAC CTG CTG AAT CTG GTA CAT G	
5'GSP-EbPKCβ-R2	GCA AGA ACA CGC TTC TCC ACC ATG GTG C	
Eb18s rRNA-RT-For	CTG CAC GCG CGC CAC ACT GAC TGG	
Eb18s rRNA-RT-Rev	CAA CGA GTG CCA CGG ACG GCC CG	
EbPKC ^{βI-} RT-For	GAC CGC TGT GAT GCT GGT AAC	Primers for
EbPKCBI-RT-Rev	CAT GGA TAA TGA ACT CAG GGT TAG TG	RT-PCR and real-time PCR
EbPKCBII-RT-For	GAG ATG CGG TCA ACT TCG ACA AG	-
EbPKCBII-RT-Rev	TCA GAG AGA GGG CCC AAA AGC	
EbPKCßI-Full-F	ACG CGG GGA GAT TGA GAA AGA G	
EbPKC _β I-Full-R	TAG CGG TAG CCT TGC ATC TTT ATC ATC	
EbPKCβI-ORF-F	ATG TCG GAG ATG GAC AGC GAG	
EbPKCβI-ORF-R	CTA AAC ATG GAT AAT GAA CTC AGG GTT AGT GA	
EcoRI-EbPKCBI-F	GGA ATT CGA TGT CGG AGA TGG ACA GC	Primers for the construction
XhoI-EbPKCßI-R	CCC TCG AGC TAA ACA TGG ATA ATG AAC TCA G	of pET32
EbPKCßII-Full-F	ACG CGG GGA GAG AAA AGA GAA GCG	_
EbPKCßII-Full-R	ACA GCT GCC AAT ATC AAC TCT AAA GCC TAG C	
EbPKCBII-ORF-F	ATG TCG GAG ATG GAC AGC GAG CAT TTG	
EbPKCBII-ORF-R	TCA GAG AGA GGG CCC AAA AGC CGG	
XhoI-EbPKCBII-R	CCT CGA GTC AGA GAG AGG GCC C	

<Table 1> and employed in order to amplify cDNAs from the inshore hagfish cDNA library.

3' cDNA library screening was conducted with GSP (gene specific primer) (sense primer, 3 3'GSP-EbPKC β -F1 and 3'GSP-EbPKC β -F2, Table 1) and Universal primers. In order to isolate the EbPKC β , 5′ full-length RACE-PCR was conducted using the GeneRacer[™] Kit (Invitrogen, Korea). 5' RACE-PCR clone was amplified with gene specific primer (antisense primer, 5'GSP-EbPKC β -R1 5'GSP-EbPKC β -R2, and <Table 1> and Universal primers. Next, the PCR product was recovered by GeneAll® SV gel (GeneAll, Korea). The purified products were then ligated into pGEM T-Easy vector (Promega, Korea). Finally, database searches were performed using the BLAST (Basic Local Alignment Search Tool) at the NCBI (National Center for Biotechnology Information).

2. Sequence and phylogenetic analysis

Nucleotide and predicted peptide sequences of E. burgeri PKC βI and βII (EbPKC βI and EbPKC β 11) were analyzed using DNASIS for Windows version 2.5 (Hitachi software engineering Co., Japan), BioEdit Sequence Alignment Editor, and BLAST programs in the non-redundant databases of the NCBI (http://www.ncbi.nlm. nih.gov/BLAST/). Multiple alignments of $EbPKC\beta I$ and βII amino acid sequences were analyzed using CLUSTAL W version 1.8. The identities and homologies between amino acid sequences were analyzed using BioEdit Sequence Alignment Editor version 5.0.9. The multiple sequence alignment obtained was used in order to generate a phylogenetic tree using neighbor-joining methods, and the reliability of the trees was evaluated using the bootstrap method

with 1,000 replications. In order to identify possible phylogenetic clade, a neighbor-joining tree was generated based on this genetic distance matrix using Kimura 2-parameter model included in MEGA 6.

3. Tissue specific expression patterns of EbPKC β I and β II by qRT-PCR analysis

were measured by qPCR analysis. The total RNA extraction and reverse transcription was obtained through the same process described above (2). For the tissue distributions of *EbPKC* β *I* and β *II*, 18s rRNA was used as an internal control gene. The specific primers Eb18s-rRNA-RT-F, Eb18s-rRNA-RT-R, EbPKC β I-RT-F, EbPKC β I-RT-F, EbPKC β II-RT-F, and EbPKC β II-RT-R were used for qPCR(<Table 1>).

EDPRC β and β if by qR1-PCR analysis The mRNA distribution of *EbPKC \betaI and \betaII*

A CGC GGG GAG AGA AAA GAG AAG CGG CTA AGA GGA GGT CGA CGT GGA CTG 49 GAG GAG CGA AGG GAA GAC GAG GTC GAG GTC GAG GTC GAG GTC GAG GTT GTG 100 GTT GTC GGC GCT TCG GAG AAA TAA ATT TGG AGT GCG GTG GAG GAA GTA AAG 151 AGG AGT GCG AGG CAG TTT GAT TGT TTC CTC CTC GAG TGT CTG TTT ATC 202 CAA CGC CGA GAC ACC AAG ACG GTC GGA ATC GCG ATG TCG GAG ATG GAC AGC 253 1-1 S F 5.4 D GAG CAT TTG GCG GAG CCT GGC GAG ACG CGC TTC GCG CGA CAA GGA GCC GTG 304 A E P G E T B F A B O G . AAA CAC AAG AAC GTG CAC GAG GTG AAG GAC CAC AAG TTC ATC GCG CGC TTC 355 TTC AAA CAG CCC ACG TTC TGC AGC CAC TGC AAG GAC TTC ATC TGG GGA TTT F K Q P T F C S H C K D F I W G F GGA AAG CAA GGC TTT CAG TGT CAA GTG TGC AGC TTT GTG GTC CAC AAG AGA 457 TGC CAC GAG TTT GTG ACA TTC TGC TGT CCA GGT GCA GAC AAA GGG GCA GAC 508 ACT GAT GAT CCA CGG AGC AAG CAC AAG TTC AAG GCC CAC ACT TAC AGT GGG 559 CCA ACC TTT TGC GAT CAC TGC GGC TCA TTG CTC TAC GGC CTT TTG CAC CAG 610 GGC ATG ANG TGC GAC ACT TGC GAG ATG AAC GTC CAT ANG GGG TGT CAG CAG 661 AAT GTT CCA AGC CTA TGC GGA ATG GAT CAC ACG GAA CGT CGA GGC CGA ATC 712 <mark>s l c</mark> g m d G R н т N ATC TAC ATT GTG GCA GAA GTC ACT GGT CCT CAT ACG CTT CAC GTC ACA GTG 763 I Y I V A E V T G P H T L H V T V AAG GAG GCT CGT AAC CTG ATC CCC ATG GAC CCG AAT GGC CTC TCG GAT CCT 814 K E A R N L I P M D P N G L S D P TAT GTG AAG CTG AAG CTG GTG CCG GAC CCG AAG AAC GAG AGC AAG CAG AAA 865 Y V K L K L V P D P K N E S K Q K ACA AAA ACT ATC AAG GCA ACA CTG AAT CCG ATG TGG CAA GAA AAC TTC TCT N 916 к TTC AAG CTG AAG GGA GAG GAT AGT GAC CGA AGG CTT TCG ATT GAG GTG TGG 967 GAC TGG GAC CGG ACT ACT CGG AAT GAC TTC ATG GGT TCA ATG TCC TTT GGT 1018 GTT TCA GAA CTG GTG AAA TGC CAA GCA GAT GGC TGG TAC AAG CTT CTG AGC 1069 D G CAA GAA GAA GGG GAG TAT TAC AAC GTT CCC GTT GCA CCC GAG GGA GAG GAA 1120 \sim E Y \sim G E X N P A P E G E E GGC CTT GAA CTT CGA CAA AGA CTG CAG AGA TCA CAA ATT GGC CGC AGC AGC 1171 S 1.2 F 1.12 B Q B 0 R Q 11 G B S AAG GGG AAA CAG AGC CCA GCA GAA AAT CAA CCG AGT GAG AGT CGA TCG TTG 1222 E N P Q S A Q S E S R 1273 L D B VKLEDF TEL G S GGA AAA GGG AGC TTT GGC AAG GTG ATG CTT GCC GAG CAG AAA AAT ACA CAA 1324 G K

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CAG	CTG	TAC	GCC	ATC	AAG	ATC	CTG	AAG	AAG	GAC	GTA	GTT	ATC	CAG	GAT	GAT	1375
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GAT	GTG	GAG	TGC	ACC	ATG	GTG	GAG	AAG	CGT	GTT	CTT	GCC	стт	тсс	AAC	AAG	1426
D	V	E	С	т	M	V	E	K	R	V	L	A	L	S	N	ĸ	
CCA	CCG	ттт	CTC	ACA	GCA	TTG	CAT	тст	TGC	TTC	CAG	ACG	AAT	GAC	CGG	CTG	1477
P	P	F	L	т	A	L	н	S	C	F	Q	Т	N	D	R	L	
TAC	TTT	GTG	ATG	GAG	TTC	GTG	AAT	GGA	GGT	GAT	CTC	ATG	TAC	CAG	ATT	CAG	1528
Y	F	V	M	E	F	V	N	G	G	D	L	м	Y	Q	I.	Q	
CAG	GTC	AGG	CGC	TTC	AAG	GAG	CCC	CAT	GTT	GTT	TTC	TAT	GCA	GCT	GAA	ATT	1579
Q	V	B	B	F	K	E	Р	н	V	V	F	Y	A	A	E	1	
GCT	GTG	GGG	TTA	CTC	TAC	TTG	CAC	GGA	AAG	GGA	ATA	ATT	TAC	AGG	GAC	CTC	1630
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AAG	CTG	GAC	AAC	GTA	ATG	TTG	GAC	TCG	GAA	GGC	CAC	ATC	AAA	ATT	GCT	GAC	1681
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TTT	GGG	ATG	TGC	AAG	GAG	AAT	GTT	TGG	GAA	GGA	ATT	ACA	ACG	CGC	ACA	TTC	1732
F	G	м	C	K	E	N	V	W	E	G	1	т	т	R	т	F	
TGT	GGA	ACT	CCA	GAC	TAT	ATC	GCT	CCT	GAG	ATA	ATA	GCA	TAC	CAA	CCT	TAT	1783
C	G	Т	P	D	Y	1	A	P	E	1	1	A	Y	Q	P	Y	
GGT	CAA	TCT	GTG	GAC	TGG	TGG	GCA	TAT	GGA	GTC	TTG	CTG	TAT	GAG	ATG	TTG	1834
G	Q	S	V	D	W	W	A	Y	G	V	L	L	Y	E	м	L	
GCT	GGA	GAG	CCG	CCA	ттт	GAT	GGT	GAA	GAT	GAG	GAT	GAG	CTT	TTC	CAG	TCA	1885
A	G	E	P	P	F	D	G	E	D	E	D	E		F	Q	S	
ATC	ATG	GAG	CAC	AAT	GTG	CCG	TAC	CCC	AGG	TTC	ATG	TCC	AAG	GAA	GCC	ATC	1936
1	м	E	н	м	V	P	Y	P	R	F	м	S	ĸ	E	A	- E	
тст	GTC	TGC	AAA	GGG	CTT	TTG	AAC	AAG	CAC	CCC	GCA	AAG	CGA	TTG	GGT	TGT	1987
S	\sim	С	ĸ	G	L.	L	N	к	н	P	A	K	R	L	G	С	
GGG	CCC	GAG	GGA	GAG	CAG	GAC	ATT	CGT	GAG	CAC	CTG	TTC	ттт	CGT	CAC	ATT	2038
G	P	E	G	E	Q	D	1	R	E	н	L	F	F	R	н	1	
GAC	TGG	GAG	CGC	CTT	GAG	CAG	CGG	GAA	ATC	CAG	CCA	CCG	TTC	AAG	CCA	AAA	2089
D	w	E	в		E	Q	8	V5 de	main		P	P	F	ĸ	P	K	
CTG	AAA	GAC	CGC	TGT	GAT	GCT	GGT	AAC	TTC	GAC	AGG	GCC	TTC	ACC	ACT	GAT	2140
L	к	D	R	С	D	A	G	N	F	D	R	A	F	т	т	D	
тст	GTC	ACG	ATG	ACG	CCG	GTG	GAC	CGC	TTG	CTG	GTC	стс	ACT	CTG	GAC	CAG	2191
S	\sim	т	м	т	P	V	D	R	L	L	V	L	т	L	D	Q	
AGT	GAC	TTC	GCA	GGC	ттт	тсс	TTC	ACT	AAC	CCT	GAG	TTC	ATT	ATC	CAT	GTT	2242
S	D	F	A	G	F	S	F	т	N	P	E	F	1	1	н	V	
TAG	TTC	TGC	AAC	CCA	ATT	ATA	ATG	TTC	ATG	TTA	TTG	GTT	ATA	тст	GGT	AAT	2293
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ATC	TTG	ТАА	AGA	CAG	TTC	AGC	TGA	TAC	TGG	ACA	CCT	TCA	CAA	GCT	CAA	AAC	2344
TTG	CAT	GTG	GAA	AAA	TAT	ATT	TCC	AAA	AAA	GCT	TAA	CAA	CAA	CCA	CTT	AAC	2395
GAC	TTA	ттт	TGG	GAT	GCT	TTC	AAA	AAA	AGA	GGG	TAC	GCA	TGT	GGT	GAT	GAT	2446
GAT	GAT	AAA	GAT	GCA	AGG	CTA	CCG	CTA	AAA	AAA	AAA	AAA	AAA	AAA	ААА	AAA	2497
AA	3.																2499

[Fig. 1] Nucleotide sequence and deduced amino acid sequence of EbPKC \$1.

Shaded sequences indicate C1, C2, Kinase, and V5 domains. The four circles are lipid cofactor binding surface inside the C1 domain. The two squares are calcium-binding Asp and the square brackets are RACK binding sites. The three triangles indicate ATP binding site (GXGXXG). In the Kinase domain, the first circle is invariant Lys and the second circle is Met as a gatekeeper residue. An underlined Trp is the turn motif and an underlined Ser is the hydrophobic motif. Asterisk (*) at the end of amino acid sequence shows the stop codon.

LightCycler® 480 SYBR Green I Master (Roche, Switzerland) was used in order to monitor the quantitative real-time PCR of mRNA transcript abundance on the LightCycler® 480 II Real-Time PCR System (Roche) using the following program: pre-incubation at 95°C for 5 min, 30 cycles at 95°C for 10 s, 60°C for 10 s, and 72°C for 10 s. The qPCR mixture was made up of the following components: 10 μ l of 2X SYBR (Roche), 7.5 μ l of SYBR water (Roche), 1 μ l of sense primer, 1 μ l of antisense primer, and 0.5 μ l diluted first-strand cDNA (diluted at 1:20)

The $^{\varDelta \Delta CT}$ method was adopted in order to calculate the data and the $2-^{\varDelta \Delta Ct}$ method was adopted in order to calculate the relative quantitative value (Giulietti et al, 2001).

4. Expression and purification of recombinant EbPKC *J* and *J* in *E. coli*

In order to construct an expression vector for the suitable production of recombinant *EbPKC* βI and βII in E. coli, the open reading frame (ORF) of *EbPKC* βI was amplified by PCR using the primers (sense primer, EcoRI-EbPKC β I-F; and antisense primer XhoI-EbPKC β I-R, as shown in <Table 1>).

A CGC GGG GAG ATT GAG AAA GAG AAG CGG CTA AGA GGA GGT CGA CGT GGA 40 CTG GAG GAG CGA AGG GAA GAC GAG GTC GAG GTC GAG GTC GAG GTC GAG GTT 100 GTG GTT GTC GGC GCT TCG GAG AAA TAA ATT TGG AGT GCG GTG GAG GAA GTA 151 AAG AGG AGT GCG AGG CAG TTT GAT TGT TTC CTC CTC CCC GAG TGT CTG TTT 202 ATC CAA CGC CGA GAC ACC AAG ACG GTC GGA ATC GCG ATG TCG GAG ATG GAC 253 64 S F M D AGC GAG CAT TTG GCG GAG CCT GGC GAG ACG CGC TTC GCG CGA CAA GGA GCC 304 EHLAEPGETRFA S B Q G A GTG AAA CGC AAG AAC GTG CAC GAG GTG AAG GAC CAC AAG TTC ATC GCG CGC 255 C1 domain H E V K KRKNV н D К F 14 TTC TTC AAA CAG CCC ACG TTC TGC AGC CAC TGC AAG GAC TTC ATC TGG GGA 406 FKQPTFCSHCKDFI W GGA AAG CAA GGC TTT CAG TGT CAA GTG TGC AGC TTT GTG GTC CAC GAG 457 G K Q G F Q C Q V C S F V V H AGA TGC CAC GAG TTT GTG ACA TTC TGC TGT CCA GGT GCA GAC AAA GGG GCA 508 R C H E F V T F C C P G A D K G A GAC ACT GAT GAT CCA CGG AGC AAG CAC AAG TTC AAG GCC CAC ACT TAC AGT н D T D D P R S K H K F K A Т GGG CCA ACC TTT TGC GAT CAC TGC GGC TCA TTG CTC TAC GGC CTT TTG CAC 610 т ғ с р н с д ѕ ∟ ∟ (Ү) д (∟ G L CAG GGC ATG AAG TGC GAC ACT TGC GAG ATG AAC GTC CAT AAG GGG TGT CAG 661 C E M M C D N н CAG AAT GTT CCA AGC CTA TGC GGA ATG GAT CAC ACG GAA CGT CGA GGC CGA 712 C2 domain V P S L C G M D H T G R N TAC ATT GTG GCA GAA GTC ACT GGT CCT CAT ACG CTT CAC GTC ACA GTG AAG 763 GAG GCT CGT AAC CTG ATC CCC ATG GAC CCG AAT GGC CTC TCG GAT CCT TAT 814 M D D N GTG AAG CTG AAG CTG GTG CCG GAC CCG AAG AAC GAG AGC AAG CAG AAA ACA 865 AAA ACT ATC AAG GCA ACA CTG AAT CCG ATG TGG CAA GAA AAC TTC TCT TTC 916 ANG CTG ANG GAG GAG AGT AGT GAC CGA AGG CTT TCG ATT GAG GTG TGG GAC 967 TGG GAC CGG ACT ACT CGG AAT GAC TTC ATG GGT TCA ATG TCC TTT GGT GTT 1018 TCA GAA CTG GTG AAA TGC CAA GCA GAT GGC TGG TAC AAG CTT CTG AGC CAA 1069 GAN GAN GGG GAG TAT TAC AAC GTT CCC GTT GCA CCC GAG GGA GAG GAA GGC 1120 N -G E 21 P 11 0 0 E G -CTT GAA CTT CGA CAA AGA CTG CAG AGA TCA CAA ATT GGC CGC AGC AGC AAG 1171 E 1 B Q B -Q B S Q 1.1 G B S S GGG AAA CAG AGC CCA GCA GAA AAT CAA CCG AGT GAG AGT CGA TCG TTG AGT 1222 B s Q E Q E s S 1 GGT CTG GAT CGT GTT AAG CTG GAA GAC TTC ACC TTC CTC ACA GTG TTG GGA 1273 24 D B K -E D F F L. AAA GGG AGC TTT GGC AAG GTG ATG CTT GCC GAG CAG AAA AAT ACA CAA CAG 1324 10 6

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CT	G TA	c GCC	ATC	AAG	ATC	CTG	AAG	AAG	GAC	GTA	GTT	ATC	CAG	GAT	GAT	GAT	1375
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GT	G GA	G TGC	ACC	ATG	GTG	GAG	AAG	CGT	GTT	CTT	GCC	CTT	TCC	AAC	AAG	CCA	1426
V	E	C	т	м	V	E	к	R	\sim	L	A	L	S	N	К	P	
CC	а тт	т сто	C ACA	GCA	TTG	CAT	TCT	TGC	TTC	CAG	ACG	AAT	GAC	CGG	CTG	TAC	1477
P	F	L	т	A	E	н	S	C	F	Q	т	N	D	R	L	Y	
TT	T GT	G ATC	GAG	TTC	GTG	AAT	GGA	GGT	GAT	CTC	ATG	TAC	CAG	ATT	CAG	CAG	1528
F	V	M) Е	F	V	ы	G	G	D	L	м	Y	Q	T	Q	Q	
GT	C AG	G CGC	> TTC	AAG	GAG	ccc	CAT	GTT	GTT	TTC	TAT	GCA	GCT	GAA	ATT	GCT	1579
×	R	R	F	K	E	P	н	~	~	F	Y	A	A	E		A	
GIU	a aa	GIIA	A CIC	TAC	T I G	CAC	GGA	AAG	GGA	AIA	ATT	TAC	AGG	GAC	CIC	AAG	1630
CT.	G			ATC	TIC	H	TCC	CAA	G	CAC	ATC		ATT	COT	CAC	TTT	160.1
CI	G GA	C AAC	- GIA	AIG	110	GAC	- CG	GAA	GGC	CAC	AIC	~~~~	ALL	GCT	GAC	=	1081
GG	G AT	G TO	- AAG	GAG	AAT	GTT	TGG	GAA	CGA	ATT	ACA	ACG	CGC	ACA	TTC	TGT	1732
G	M	C IC	K	F	N	V	w	F	G	~	т	т	B	T	F	C	17.02
GG	A AG	TCCA	GAC	TAT	ATC	GCT	CCT	GAG	ATA	ATA	GCA	TAC	CAA	CCT	TAT	GGT	1783
G	т	P	D	Y		A	P	E	1		A	Y	Q	P	Y	G	
CA	A TO	T GTO	GAC	TGG	TGG	GCA	TAT	GGA	GTC	TTG	CTG	TAT	GAG	ATG	TTG	GCT	1834
Q	S	V	D	w	w	A	Y	G	\sim	L	- L	Y	E	м	L.	A	
GG	A GA	G CCC	G CCA	TTT	GAT	GGT	GAA	GAT	GAG	GAT	GAG	CTT	TTC	CAG	TCA	ATC	1885
G	E	P	P	F	D	G	E	D	E	D	E		F	Q	S	1.	
AT	G GA	GCAC	- AAT	oto	666	TAC	000	100	TTC	ATC	TCC	0.00	GAA	000	ATC	TOT	1936
	· ·	a one		GIG	000	1740	ccc	AGG	110	AIG	100	AAG	Contra	acc	AIC	101	1000
м	E	н	N	v	P	Y	P	R	F	M	s	K	E	A	i	s	
M GTC	E	н	N	CTT	P	Y AAC	P	R	F	M	S	K	E	A	тат	S	1987
м GTC V	E TGC C	H AAA K	N GGG G		P TTG L	AAC N	P AAG K	R CAC H	F CCC P	M GCA	S AAG K	K CGA B	E TTG L	A GGT G	і тат с	s GGG G	1987
M GTC V CCC	E TGC C GAG	H AAA K GGA	N GGG G GAG		P TTG L GAC	AAC N ATT	P AAG K CGT	R CAC H GAG	F CCC P CAC	M GCA A CTG	S AAG K TTC	K CGA R TTT	E TTG L CGT	A GGT G CAC	TGT C ATT	S GGG G GAC	1987
M GTC V CCC P	E TGC C GAG E	H AAA K GGA G	N GGG G GAG E		P TTG L GAC D	AAC N ATT	P AAG K CGT R	R CAC H GAG E	F CCCC P CAC	M GCA A CTG L	S AAG K TTC F	K CGA R TTT F	E TTG L CGT R	A GGT G CAC H	I TGT C ATT	S GGG G GAC D	1987
M GTC V CCC P TGG	E TGC C GAG E GAG	H AAA K GGA G CGC	N GGG G GAG E CTT		P TTG GAC D CAG		P AAG K CGT R GAA	R CAC H GAG E ATC	F CCCC P CAC H	M GCA A CTG L CCA	AAG K TTC F CCG	K CGA R TTT F TTC	E TTG L CGT R AAG	A GGT G CAC H CCA	I TGT C ATT I AAA	S GGG GAC D CTG	1987 2038 2089
M GTC V CCC P TGG W	E TGC C GAG E GAG E	H AAA K GGA G CGC R	N GGG GAG E CTT L		P TTG GAC D CAG	AAC N ATT I CGG	P AAG K CGT R GAA S don E	R CAC H GAG E ATC		M GCA A CTG L CCA	S AAG K TTC F CCG P	K CGA R TTTT F TTC	E TTG L CGT R AAG	A GGT G CAC H CCA	I TGT C ATT I AAA	S GGG GAC D CTG	1987 2038 2089
M GTC V CCCC P TGG W TGT	E TGC C GAG E GAG E GGC	H AAA K GGA G CGC R CGA	N GGG GAG E CTT L GAT	CTT L CAG GAG E GCG	P TTG GAC D CAG Q GTC	AAC N ATT I CGG R AAC	P AAG K CGT R GAA E TTC	R CAC H GAG E ATC I GAC		M GCA A CTG L CCA P TGT	S AAG K TTC F CCG P TTC	K CGA R TTT F TTC F ACG	E TTG L CGT R AAG K CGA	A GGT G CAC H CCA P GTA	I TGT C ATT I AAA K CAA	S GGG GAC D CTG L CCG	1987 2038 2089 2140
M GTC V CCCC P TGG W TGT C	E TGC GAG E GAG E GAG E GGC	H AAA K GGA G CGC R CGA	N GGG GAG E CTT L GAT D	CTT L CAG GAG E GCG A	P TTG GAC D CAG Q GTC	AAC N ATT I CGG R AAC	P AAG K CGT R GAA S dom E TTC	R CAC H GAG E ATC I GAC D	F CCCC P CAC H CAG AAG	M GCA A CTG L CCA P TGT C	S AAG K TTC F CCG P TTC F	K CGA R TTT F TTC F ACG T	E TTG L CGT R AAG K CGA R	A GGT G CAC H CCA P GTA	I TGT C ATT I AAA K CAA Q	S GGG GAC D CTG CTG L CCG	1987 2038 2089 2140
M GTC V CCC P TGG TGG TGT GTC	E TGC GAG E GAG E GAG CTC	H AAA K GGA G CGC R CGA R ACA	N GGG GAG E CTT L GAT D CCG	CTT L CAG GAG E GCG A CCT	P TTG L GAC D CAG GTC Q GAT		P AAG K CGT R GAA E TTC F TAC	R CAC H GAG E ATC Jain I GAC GAC	F CCC P CAC H CAG AAG K ATC	M GCA A CTG L CCA P TGT C AAC	S AAG K TTC F CCG P TTC F AAC	K CGA R TTT F TTC F ACG T ATC	E TTG L CGT R AAG K CGA R GAC	A GGT G CAC H CCA P GTA V CAG	I TGT C ATT I AAA K CAA Q TCT	S GGG GAC D CTG CTG CCG P GAG	1987 2038 2089 2140 2191
M GTC V CCC P TGG W TGT GTC V	E TGC C GAG E GAG E GGC G CTC L	H AAA K GGA G CGC R CGA R ACA T	N GGG GAG E CTT L GAT D CCG	CTT L CAG GAG E GCG A CCT P	P TTG L GAC D CAG GTC Q GAT D		P AAAG K CGT R GAA E TTC F TAC Y	R CAC H GAG E ATC GAC D GTC V	F CCCC P CAC H CAG AAG K ATC	M GCA A CTG L CCA P TGT C AAC N	AAG K TTC F CCG P TTC F AAC N	K CGA R TTT F TTC F ACG T ATC I	E TTG L CGT R AAG K CGA R GAC D	A GGT G CAC H CCA P GTA V CAG Q	I TGT C ATT I AAA K CAA Q TCT S	S GGG GAC D CTG CTG CCG GAG E	1987 2038 2089 2140 2191
M GTC V CCC P TGG W TGT GTC Q TTT	E TGC C GAG E GAG E GGC CTC L GGC	H AAA K GGA G CGC R CGA R ACA T GGC	N GGG GAG E CTT L GAT D CCG P TTT	CTT C	P TTG L GAC D CAG GTC V GAT D TAC	AAC N AATT I CGG R AAC N GAA E GTG	P AAG K CGT R GAA S dom E TTC F TAC Y AAC	R CAC H GAG E ATC GAC GAC GTC V CCG	F CCCC P CAC H CAG Q AAG K AAG K ATC I GCT	M GCA A CTG CCA P TGT C AAC N TTT	S AAG K TTC F CCG P TTC F AAC N GGG	K CGA R TTT F TTC F ACG T ATC I CCC	E TTG L CGT R AAG K CGA R GAC D TCT	A GGT G CAC H CCA GTA V CAG Q CTC	I TGT C ATT I AAA K CAA Q TCT S TGA	S GGG G GAC D CTG CCG P GAG E ACA	1987 2038 2089 2140 2191 2242
M GTC V CCC P TGG W TGT GTC Q TTT F	E TGC C GAG E GAG E GGC CTC L GGC G	H AAA GGA GGC R CGA R ACA T GGC G	N GGG GAG E CTT L GAT D CCG P TTT F	CTT CAG Q QAG GAG CCT CCT CCT CCT CCT CCT CCT CC	P TTG L GAC D CAG GTC V GAT D TAC Y	AAC N ATT I CGG R AAC N GAA GAA E GTG	P AAG K CGT R GAA <u>S</u> dom E TTC F TAC Y AAC N	R CAC H GAG E ATC GAC GAC GTC V CCG P	F CCCC P CAC H CAG Q AAG K AAG K ATC I GCT A	M GCA A CTG CCA P TGT C AAC N TTT F	S AAG K TTC F CCG P TTC F AAC N GGG	K CGA R TTT F TTC F ACG T ATC I CCC P	E TTG L CGT R AAG K CGA R GAC D TCT S	A GGT G CAC H CCA GTA V CAG Q CTC L	I TGT C ATT I AAA K CAA Q TCT S TGA *	S GGG GAC D CTG CTG CCG GAG GAG E ACA	1987 2038 2089 2140 2191 2242
M GTC V CCCC P TGG W TGT GTC V TTT F TGG	E TGC GAG E GAG E GGC CTC L GGC G CTA	H AAA GGA GGC R CGA R ACA T GGC G GAT	N GGG GAG E CTT L GAT D CCG P TTT F CCA	CTT L CAG GAG GAG GAG CT CCT CCT CCT CCT CCT CCT CCT CCT CCT	P TTG L GAC D CAG GTC V GAT D TAC Y CAA	AAC N ATT I CGG R AAC N GAA GAA E GTG V AGC	P AAG K CGT R GAA E TTC F TTC F TAC Y AAC N CAC	R CAC H GAG E ATC GAC D GAC D GTC V CCG P GGC	F CAC CAC CAG CAG CAG CAG CAG CAG CAG CAG	M GCA A CTG CCA P TGT C AAC N TTT F CTG	S AAG K TTC F CCG P TTC F AAC N GGG GTT	K CGA R TTT F TTC F ACG T ACG T ACC	E TTG CGT R AAG CGA R GAC D TCT S TTG	A GGT GAC H CCA P GTA V CAG GTA CTC L GCT	I TGT ATT AAA K CAA CAA TCT S TGA * CGT	S GGG GAC D CTG CTG CCG GAG GAG E ACA	1987 2038 2089 2140 2191 2242 2293
M GTC V CCCC P TGG W TGT GTC V TTT F TGG GTC	E TGC GAG E GAG E GGC G CTC L GGC G CTA TGC	H AAA K GGA G CGC R CGA R ACA GGC G GAT GGC	N GGG G GAG E CTT L GAT D CCG P TTT F CCA GTG	CTT CAG Q GAG E GAG CT C C C C C C C C C C C C C C C C C C	P TTG L GAC D CAG GTC V GAT D TAC Y CAA AAC	AAC N ATT I CGG AAC N GAA E GTG V AGC ACA	P AAG K CGT R GAA E TTC F TTC F TAC Y AAC N CAC GAT	R CAC H GAG E ATC GAC D GAC V CCG P GGC CCA	F CAC CAC CAG CAG CAG CAG CAG CAG CAG CAG	M GCA CTG CCA P TGT C AAC N TTT F CTG TAA	AAG K TTC F CCCG P TTC F AAC GGG GTT ACC	K CGA R TTT F TTC F ACG T ACG T ACC P AGC CCA	E TTG L CGT R AAG CGA R GAC D TCT S TTG GCT	A GGT GAC H CCA GTA CCA GTA CAG GTA CTC E GCT TAC	TGT C ATT I AAA CAA G TCT S TGA * CGT CGA	S GGG GAC D CTG CCG P GAG GAG ACA TGC GCC	1987 2038 2089 2140 2191 2242 2293 2344
M GTC V CCC P TGG W TGT GTC V TTT F TGG GTC CTC	E TGC GAG E GAG E GGC G CTC L GGC G CTA TGC CAA	H AAA K GGA G CGC R CGA R ACA T GGC GAT GGC CCT	N GGG GAG E CTT L GAT D CCG P TTT F CCA GTG GAC	CTT L CAG GAG E GCG CCT CCT CCT CCT CCT CCT CCT CCT CCT	P TTG GAC D CAG GTC V GAT TAC Y CAA AAC CAG	AAC N ATT I CGGG R AAC N GAA GTG V AGC ACA CAC	AAG K CGT R GAA E TTC F TTC F TAC Y AAC GAT ACC	R CAC H GAG E ATC GAC D GAC V CCG GGC CCA TGA	F CCC CAC H CAC H CAC A AAG A AAG A CAC C C C C C C C C C	M GCA CTG CCA P TGT C AAC N TTT F CTG TAA TCA	AAG K TTC F CCG P TTC F AAC GGG GTT ACC CAA	K CGA R TTT F TTC F ACG T ACG CCC AGC CCA AAC	E TTG L CGT R AAG K CGA R GAC D TCT S TTG GCT ACC	A GGT GAC H CCA P GTA V CAG GTA CTC CTC GCT TAC ACA	TGT G ATT I AAA CAA G TCT S GA CGT CGA ACC	S GGG GAC D CTG CCG P GAG GAG CA TGC GCC TAA	1987 2038 2089 2140 2191 2242 2293 2344 2395
M GTC V CCC P TGG W TGT GTC V TTT F TGG GTC CTC CCT	E TGC C GAG E GAG C C C C C C C C C C C C C C C C C C	H AAA GGA G CGC R CGA R ACA T GGC GAT GGC CCT ATG	N GGG GAG E CTT L GAT O CCG P TTT F CCA GAC GAC CGC	CTT CAG Q GAG E GCT CCT CCT CCT CCT CCT CCT CCT CCT CCT	CAG GAC CAG GAC CAG GAT CAA CAA CAA CAA CAA CAA CAA CAA	AAC N ATT I CGG GG AAC GAA E GTG V AAC AGC ACA CAC	P AAG K CGT R GAA TTC F TTC F TTC AAC GAT ACC GAT ACC	R GAG GAG H GAG E ATC GAG GAG CCG P GGC CCA TGA AAC	F CAC CAC CAC H CAG AAAG K AAAG K AAAG CAC CAC H CAC CAC CAC CAC CAC	M GCA CTG CTG CCA P TGT C CCA N TTT F CTG CTG CTG CTG CTG CTG CTG CTG	AAG K TTC F CCG F TTC F AAC G G G TT ACC CAA AAT	K CGA R TTT F TTC ACC ATC I CCC P AGC ACC ACC ACC ACC	E TTG L CGT R AAG GAC CGA R GAC TCT S GGT ACC ATG	A GGT G CAC H CCA P GTA CAG CTC CAG GCT CTC L GCT TAC ACA	TGT C ATT I AAAA C C C C C C C C C C C C C C C C	S GGGG GAC D CTG CTG CTG GAG GAG F GAG GAG C GAG TGC GCC TAA TTG	1987 2038 2089 2140 2191 2242 2293 2344 2395 2446
M GTC V CCC P TGG GTC GTC GTC CTC CTC CCT CTC	E TGC GAG E GAG E GGC CTC GGC GGC CTA CTA CAA GAA TGA	H AAA K GGA GC CGA R CGA R ACA GGC GAT GGC GAT GGC CCT ATG	N acca G acca CTT L GAT CCG P TTT F CCA GTG GAC CGC TGT	CTT CAG Q GAG E GAG CCT CC C CCT C C C C C C C C C C C C C	P TTG GAC D CAG G G G TAC Y CAA CAG TTG TGA	AAC N ATT I CGG CG GAA E GAA E GAA E GAA CAC CCG TGA	P AAG K CGT R GAA F TTC F TAC Y AAC N GAA GAAT ACC TTA	R GAG GAG E GAG E GAG C GAG C GAG C C GGC C C GGC C C C C GAG C C C C C C C C C C C C C	F GCCC P GAC H CAG A A A A CAG C CAG C C A C C C C C C	M GCA CTG CTG CCA P TG CCA P TG C AAC N TTT F CTA TCA CGG TTT	S AAG K TTC F CCG P TTC F AAC N GGG GT ACC CAA AAT TTT	K CGA R TTT F TTC ACC ATC I CCC P ACC ACC ACC ACC GTG GTT	E TTG L CGT R AAG CGA GAC B TCT S GAC TCT S GGT ACC ATG TGT	A GGT G CAC H CCA P GTA CAG CTC CAG CTC C CAG CTC C CAG CTC C CAG CAC CAC CAC CAC CAC CAC CAC CAC	TGT C ATT I AAA C C AA TCT S TGA C C G TA C C G TA TTT	S GGGG GAC D CTG CTG CTG GAG GAG E ACA TGC GCC TAA TTG GTT	1987 2038 2089 2140 2191 2242 2293 2344 2395 2446 2497
M GTC V CCC P TGG GTC V TGT GTC CTC CTC CTA	E TGC C GAG E GAG E GGC CTC G GGC G CTA TGC CAA TGA TTT	H AAA K GGA G CGA R ACA T GGC GAT GGC CCT G ATG TGA	N GGG GAG E CTT L GGAT D CCG P TTT F CCA GGAC CGC TGT GCA	CTT L CAG G G G G G CCT C C C C C G G C C C C G G C C C C C C C C C C C C C	P TTG L GAC D CAG GAT CAG GAT CAA CAA CAA CAA CAA CAA CAA CAA CAA C	AAC N ATT I CGG CGG AAC N GAA E AAC N GAA CAC CAC CAC CAC CAC CAC CAC CAC CAC	P AAG K CGT R GGA F TTC F TTC F TTC F TAC N CAC GAT ACC ACC GAT TTA TGA	R GAG GAG E GAG E GAG C C G GAC C C G GGC C C C	F CCCC P CCCC P CCCC CCCC CCCC CCCC CCCC CCCC CCCC CCCC CCCC P CCCCC CCCC CCCC CCCC CCCCC CCCC CCCCC CCCCC CCCC CCCCC CCCCC CCCCC CCCCCC	M GCA GCA CTG CCA P TGT C AAC N TTT F CTG TAA TCA CGG TTT GTT	s AAG K TTC F CCG F ACC GGG G GTT ACC CAA AAT TTT TTC	K CGA R TTT F TTC F ACG T ACG CCC P AGC CCA AGC GTT AGA	E TTG L CGT R GGA GGA C D TCT S TTG GCT ACC TGT CACC	A GGT G CAC H CCAC GTA V CAG GTA V CAG GTA CTC L GCT TAC ACT TTG ATT	I TGT C ATT I A ATT I A A CCAA CCAA CCAA CCAA CCAA CCGT CCGA ACCG TTT CCGA ACCG TTT CCGA	S GGGG GAC D CTG CTG CCG P GAG GAG ACA TGC GCC TAA TTG GTT CTT	1987 2038 2089 2140 2191 2242 2293 2344 2395 2446 2497 2548
M GTC V CCCC P TGG GTC GTC GTC CTC CCT TGT TGG GTC CTA	E TGC C GAG E GAG E GGC G CTC L GGC G CTA TGC CAA GAA TGA TTT TCC	H AAA K GGA CGC R CGA ACA T GGC GAT GGC CCT ATG ATG ATG ATG ATG	N GGGG G G CTT L GAT D CCG P TTT F GAT GAC CCA GAC CGC GCA CAC CAC CCA	CTT L CAG GAG E GAG E CCT CC C C C C C C C C C C C C C C C	P TTG L GAC D CAG GAT CAG CAG CAA CAA CAA CAT TTGA CAT	Y AAC N ATT CGG GG AAC AAC AAC AGA CAC ACA CAC CGA GTC GAT GTC	P AAG K CGT R GGA TTC F TTC F TAC Y AAC GAT ACC GAT ACC TTA CAC GAT TTGA TGA	R GAG GAG E GAG E GAG C GAG GAC D GAC D GAC D GAC C C GAG C C GAG C C GAG C C GAG C C C C C C C C C C C C C	F CCCC CAC H CAC AAG AAG AAC I GCT CCT CCT CCT CCT CCT CCT CCT CCT CCT	M GCA CTG CCA CTG CCA P TGT C AAC N TTT C CTG TAA TCA CGG CTT GTT ATT	s aag k TTC F aac G G G G G TT acc caa at TTT TTC at T	K G G T T T T T T T T T T ACG T ACG T ACG T ACG C ACG C ACG T ACG ACG T ACG ACG ACG ACG ACG ACG ACG ACG	E TTG L CGT R AAG CGA R GAC D TCT S TTG GCT ATG ATG TGT CAC GGC	A GGT G CAC H CCAC GTA V CAG GTA V CAG GCT TAC ACA AAT TTG ATT CAC	I TGT C ATT I A C ATT I A C CAA C CAA C CAA C CGT C CGA A CCT G CCA C CTG C CCA	s GGGG G G C TG C C G G G G G G G G G G G	1987 2038 2089 2140 2191 2242 2293 2344 2395 2446 2446 2446 24467 2548
M GTC V CCC P TGG W TGT C GTC CTC CTC CTC CTC CTC CTC CTC C	E TGC GAG GAG E GGC GC GGC GGC GGC GGC GGC GAA GAA TGC CAA GAA TGC TGC CAA	H AAA K GGA GGC R CGA R ACA GGC GAT GGC GGT ATG GGC CCT ATG TTG CAC GCC	N GGG G G CTT L GAT CCG P TTT F GCG GAC CGC TGT GCA CAC TTA	CTT L CAG GAG E GAG C C C C C C C C C C C C C C C C C C	P TTG L GAG G GTC V GAT G TAC V CAG CAG CAG CAG CAG CAG CAG TTG CAT TTTG	AAC N ATT CGG CGG AAC AAC AAC AAC ACA CAC CCG CGG CG	P AAG K CGT GAA TTC F TTC F TTC F CAAC AAC N CAAC TTA AAC TTA TGT CCA TTG	R CAC H GAG E ATC SATC SATC CCG P CCG P CCG CCG CCG CCG C	F GCCG P GAG AAG AAAG K AAAG AAAG C GCT GCT GCT GCT GCT GCT GCT ACC GCT ACC GCT	M GCA A CTG CCA P TGT CCA P TGT CCA N TTT F CTG TTA TCA CGG TTT ATT GTA	S AAG K TTC F CCG P TTC F AAC GGG G G G G G G G G G G G G G G G G	K CGA R TTT F ACG T ACG CCA AAC GTG AGA CGC AAA	E TTG L CGT R CGA R CGA R CGA TCT S TCT S GCT ACC ACC ACC ACC ACC ACC	A GGT G CAC CCA GTA CCA GTA CAC GTA CAC ACA AAT TTT CAC AAA	TGT C ATT AAA CAA CAA CAA CCA ACC GTA TCGA ACC GTA TCTG CCA AAA	s GGGG G GGGC CTG CCG P GAG CCG P GAG CCG TAA TGC GCC TAA TTG GTT TAG AAA	1987 2038 2089 2140 2191 2242 2293 2344 2395 2446 2497 2548 2599 2650

[Fig. 2] Nucelotide sequence and deduced amino acid sequence of *EbPKC JII*. Shaded sequences indicate C1, C2, Kinase, and V5 domains. The four circles are lipid cofactor binding surface inside the C1 domain. The two squares are calcium-binding Asp and the square brackets are RACK binding sites. The three triangles indicate ATP binding site (GXGXXG). In the Kinase domain, the first circle is invariant Lys and the second circle is Met as a gatekeeper residue. An underlined Trp is the turn motif and an underlined Ser is the hydrophobic motif. Asterisk(*) at the end of amino acid sequence shows the stop codon.

In addition, in the case of $EbPKC \beta II$, ORF was amplified by PCR using the primers (sense primer, EcoRI-EbPKC β I-F and antisense primer XhoI-EbPKC β II-R, as shown in <Table 1>). They have the same nucleotide sequence at the beginning of ORF, so the same sense primer was used in both cases. The amplified fragment was cloned into the pET32b (Novagen). The recombinant plasmids (*EbPKC* β 1/pET32b and *EbPKC* β 11/pET32b) were transformed into *E. coli* strain BL21 (DE3). Transformed cells were grown in LB broth (5 ml) containing ampicillin (100 µg/ml) at 37°C for about 12 h, re-inoculated in two LB broths (5ml) containing ampicillin (100 µg/ml), and grown at 37°C until the OD₆₀₀=0.6. Only one of the two LB broths (5 ml) had IPTG

	HXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX	1
EbPRCbetaI HsPRCbetaI BtPRCbetaI OtPRCbetaI MsPRCbetaI HsPRCbetaI HsPRCbetaII BtPRCbetaII DrPRCbetaII RnPRCbetaII RnPRCbetaII	MSENDSEILLARIGGTR MADPAAGPPPSEGSTYR FARCAIR RIVNEVN HEY ANPERGPTECSHC DEIWG COGEC CVC EVVIER 7 MADPAAGPPPSEGSTYR FARCAIR RIVNEVN HEY MADPAAGPPPSEGSTYR FARCAIR RIVNEVN HEY MADPAAGPPPSEGSTYR FARCAIR RIVNEVN HEY MADPAAGPPPSEGSTYR FARCAIR RIVNEVN HEY MADPAAGPPSEGSTYR FARCAIR RIVNEVN HEY FARCAIR RIVNEVN HEY MADPAAGPPSEGSTYR FARCAIR RIVNEVN HEY FARCAIR RIVNEVN HEY FARCAIR RIVNEVN HEY FARCAIR RIVNEVN HEY MADPAAGPPSEGSTYR FARCAIR RIVNEVN HEY FARCAIR RIVNEVN HEY FARCAIR RIVNEVN HEY FARCAIR RIVNEVN HEY FARCAIR RIVNEVN HEY MADPAAGPPSEGSTYR FARCAIR RIVNEVN HEY FARCAIR RIVNEVN HEY FAR	4777377377677
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OcPRCbetaI DrPRCbetaI RnPRCbetaI RmPRCbetaI EbPRCbetaII BtPRCbetaII DcPRCbetaII DrPRCbetaII MmPRCbetaII	DRAL VE WEWDLT ENDEMGS SEG SEL SKAAV DEW FULS SEG VENVEV FE 3.5 ENDEL OK FLAAT DOOTKA 3 DRAL VE WEWDLT ENDEMGS SEG SEL SKAAV DEW FULS SEG VENVEV FE 3.5 ENDEL OK FLAAT DOOTKA 3 DRAL VE WEWDLT ENDEMGS SEG SEL SKAAV DEW FULS SEG VENVEV FE 3.5 ENDEL OK FLAAT DOOTKA 3 DRAL VE WEWDLT ENDEMGS SEG SEL SKAAV DEW FULS SEG VENVEV FE 3.5 ENDEL OK FLAAT DOOTKA 3 DRAL VE WEWDLT ENDEMGS SEG SEL SKAAV DEW FULS SEG VENVEV FE 3.5 ENDEL OK FLAAT DOOTKA 3 DRAL VE WEWDLT ENDEMGS SEG SEL SKAAV DEW FULS SEG VENVEV FE 3.5 ENDEL OK FLAAT DOOTKA 3 DRAL VE WEWDLT ENDEMGS SEG SEL SKAAV DEW FULS SEG VENVEV FE 3.5 ENDEL OK FLAAT DOOTKA 3 DRAL VE WEWDLT ENDEMGS SEG SEL SKAAV DEW FULS SEG VENVEV FE 3.5 ENDEL OK FLAAT DOOTKA 3 DRAL VE WEWDLT ENDEMGS SEG SEL SKAAV DEW FULS SEG VENVEV FE 3.5 ENDEL OK FLAAT DOOTKA 3 DRAL VE WEWDLT ENDEMGS SEG SEL SKAAV DEW FULS SEG VENVEV FE 3.5 ENDEL OK FLAAT DOOTKA 3 DRAL VE WEWDLT ENDEMGS SEG SEL SKAAV DEW FULS SEG VENVEV FE 3.5 ENDEL OK FLAAT DOOTKA 3 DRAL VE WEWDLT ENDEMGS SEG SEL SKAAV DEW FULS SEG VENVEV FE 3.5 ENDEL OK FLAAT DOOTKA 3 DRAL VE WEWDLT ENDEMGS SEG SEL SKAAV DEW FULS SEG VENVEV FE 3.5 ENDEL OK FLAAT DOOTKA 3 DRAL VE WEWDLT ENDEMGS SEG SEL SKAAV DEW FULS SEG VENVEV FE 3.5 ENDEL OK FLAAT DOOTKA 3 DRAL VE WEWDLT ENDEMGS SEG SEL SKAAV DEW FULS SEG VENVEV FE 3.5 ENDEL OK FLAAT DOOTKA 3 DRAL VE WEWDLT ENDEMGS SEG SEL SKAAV DEW FULS SEG VENVEV FE 3.5 ENDEL OK FLAAT DOOTKA 3 DRAL VE WEWDLT ENDEMGS SEG SEL SKAAV DEW FULS SEG VENVEV FE 3.5 ENDEL OK FLAAT DOOTKA 3 DRAL VE WEWDLT ENDEMGS SEG SEL SKAAV DEW FULS SEG VENVEV FE 3.5 ENDEL OK FLAAT DOOTKA 3 DRAL VE WEWDLT ENDEMGS SEG SEL SKAAV DEW FULS SEG VENVEV FE 3.5 ENDEL OK FLAAT DOOTKA 3 DRAL VE WEWDLT ENDEMGS SEG SEL SKAAV DEW FULS SEG VENVEV FE 3.5 ENDEL OK FLAAT DOOTKA 3 DRAL VE WEWDLT ENDEMGS SEG SEL SKAAV DEW FULS SEG VENVEV FE 3.5 ENDEL OK FLAAT DOOTKA 3	16266266366
Eb PRChetaI HaPRChetaI OcPRChetaI DrPRChetaI DrPRChetaI MmPRChetaI HaPRChetaII Go PRChetaII Go PRChetaII MmPRChetaII MmPRChetaII	EXEMPNOL DESERS LEGLOR VILLOP TO VLORGSFORVILLE EK TO LLA RILKELVVIQDOLVECTVVEKEVIAL S PERETTNIT SK DINKONEDE KLIDE TI VLORGSFORVILSE K TO LLA RILKKLVVIQDOLVECTVVEKEVIAL S PERETTNITSK DINKONEDE KLIDE TI VLORGSFORVILSE K TO LLA RILKKLVVIQDOLVECTVVEKEVIAL S PERETTNITSK DINKONEDE KLIDE TI VLORGSFORVILSE K TO LLA RILKKLVVIQDOLVECTVVEKEVIAL S PERETANTSK DISK DANON DE KLIDE TI VLORGSFORVILSE K TO LLA RILKKLVVIQDOLVECTVVEKEVIAL S PERETANTSK DISK DANON DE KLIDE TI VLORGSFORVILSE K TO LLA RILKKLVVIQDOLVECTVVEKEVIAL S PERETANTSK DISK DANON DE KLIDE TI VLORGSFORVILSE K TO LLA RILKKLVVIQDOLVECTVVEKEVIAL S PERETANTSK DISK DINKONEDE KLIDE TI VLORGSFORVILSE K TO LLA RILKKLVVIQDOLVECTVVEKEVIAL S PERETANTSK DINKONEDE KLIDE TI VLORGSFORVILSE K TO LLA RILKKLVVIQDOLVECTVVEKEVIAL S PERETANTSK DINKONEDE KLIDE TI VLORGSFORVILSE K TO LLA RILKKLVVIQDOLVECTVVEKEVIAL S PERETANTSK DINKONEDE KLIDE TI VLORGSFORVILSE K TO LLA RILKKLVVIQDOLVECTVVEKEVIAL S PERETANTSK DINKONEDE KLIDE TI VLORGSFORVILSE K TO LLA RILKKLVVIQDOLVECTVEKEVIAL S PERETANTSK DINKONEDE KLIDE TI VLORGSFORVILSE K TO LLA RILKKLVVIQDOLVECTVEKEVIAL S PERETANTSK DINKONEDE KLIDE TI VLORGSFORVILSE K TO LLA RILKKLVVIQDOLVECTVEKEVIAL S PERETANTSK DINKONEDE KLIDE TI VLORGSFORVILSE K TO LLA RILKKLVVIQDOLVECTVEKEVIAL S PERETANTSK DINKONEDE KLIDE TI VLORGSFORVILSE K TO LA RILKKLVVIQDOLVECTVEKEVIAL S PERETANTSK DINKONEDE KLIDE TI VLORGSFORVILSE K TO LA RILKKLVVIQDOLVECTVEKEVIAL S PERETANTSK DINKONEDE KLIDE TI VLORGSFORVILSE K TO LA RILKKLVVIQDOLVECTVEKEVIAL S PERETANTSK DINKONEDE KLIDE FLI VLORGSFORVILSE K TO LA RILKKLVVIQDOLVECTVEKEVIAL S PERETANTSK DINKONEDE KLIDE FLI VLORGSFORVILSE K TO LA RILKKLVVIQDOLVECTVEKEVIAL S PERETANTSK DINKONEDE KLIDE FLI VLORGSFORVILSE K TO LA RILKKLVVIQDOLVECTVEKEVIAL S PERETANTSK DINKONEDE KLIDE FLI VLORGSFORVILSE K TO LA RILKKLVVIQDOLVECTVEKEVIAL S PERETANTSK DINKONEDE KLIDE FLI VLORGSFORVILSE K TO LA RILKKVVIQDOLVECTVEKEVIAL S	93 96 92 96 92 96 92 96 92 96 96 96 96 96 96 96 96 96 96 96 96 96
EDPRCDetaI HaPRCDetaI DOPRCDetaI DOPRCDetaI DRDRCDetaI HaPRCDetaII HaPRCDetaII DCPRCDetaII DCPRCDetaII MmPRCDetaII MmPRCDetaII	SS RPPFIT LISCFOT DRLYFVMEYNGGDIN GGDIN 100VC PREPIVFYA EIAYGL 120RG IYRDIRLDNVMLDS 4 PRPFFIT LISCFOT DRLYFVMEYNGGDIN 100VC PREPIVFYA EIAIGL 120RG IYRDIRLDNVMLDS 4 PRPFFIT LISCFOT DRLYFVMEYNGGDIN 100VC PREPIVFYA EIAIGL 120RG IYRDIRLDNVMLDS 4 SS RPPFIT LISCFOT DRLYFVMEYNGGDIN 100VC PREPIVFYA EIAIGL 120RG IYRDIRLDNVMLDS 4 SS RPFFIT LISCFOT DRLYFVMEYNGGDIN 100VC PREPIVFYA EIAIGL 120RG IYRDIRLDNVMLDS 4 SS RPFFIT LISCFOT DRLYFVMEYNGGDIN 100VC PREPIVFYA EIAIGL 120RG IYRDIRLDNVMLDS 4 SS RPFFIT LISCFOT DRLYFVMEYNGGDIN 100VC PREPIVFYA EIAIGL 120RG IYRDIRLDNVMLDS 4 SS RPFFIT LISCFOT DRLYFVMEYNGGDIN 100VC PREPIVFYA EIAIGL 120RG IYRDIRLDNVMLDS 4 SS RPFFIT LISCFOT DRLYFVMEYNGGDIN 100VC PREPIVFYA EIAIGL 120RG IYRDIRLDNVMLDS 4 SS RPFFIT LISCFOT DRLYFVMEYNGGDIN 100VC PREPIVFYA EIAIGL 120RG IYRDIRLDNVMLDS 4 SS RPFFIT LISCFOT DRLYFVMEYNGGDIN 100VC PREPIVFYA EIAIGL 120RG IYRDIRLDNVMLDS 4 SS RPFFIT LISCFOT DRLYFVMEYNGGDIN 100VC PREPIVFYA EIAIGL 120RG IYRDIRLDNVMLDS 4 SS RPFFIT LISCFOT DRLYFVMEYNGGDIN 100VC PREPIVFYA EIAIGL 120RG IYRDIRLDNVMLDS 4 SS RPFFIT LISCFOT DRLYFVMEYNGGDIN 100VC PREPIVFYA EIAIGL 120RG IYRDIRLDNVMLD 4 SS RPFFIT LISCFOT DRLYFVMEYNGGDIN 100VC PREPIVFYA EIAIGL 120RG IYRDIRLDNVMLD 4 SS RPFFIT LISCFOT DRLYFVMEYNGGDIN 100VC PREPIVFYA EIAIGL 120RG IYRDIRLDNVMLD 4 SS RPFFIT LISCFOT DRLYFVMEYNGGDIN 100VC PREPIVFYA EIAIGL 120RG IYRDIRLDNVMLD 4 SS RPFFIT LISCFOT DRLYFVMEYNGGDIN 100VC PREPIVFYA EIAIGL 120RG IYRDIRLDNVMLD 4 SS RPFFIT LISCFOT DRLYFVMEYNGGDIN 100VC PREPIVFYA EIAIGL 120RG IYRDIRLDNVMLD 4 SS RFFFIT LISCFOT DRLYFVMEYNGGDIN 100VC PREPIVFYA EIAIGL 120RG IYRDIRLDNVMLD 4 SS RFFFIT LISCFOT DRLYFVMEYNGGDIN 100VC PREPIVFYA EIAIGL 120RG IYRDIRLDNVMLD 4 SS RFFFIT LISCFOT DRLYFVMEYNGGDIN 100VC PREPIVFYA EIAIGL 120RG IYRDIRLDNVMLD 4	73676672667266736673667366736
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Eb PKChetaI Ha PKChetaI OoPKChetaI DrPRChetaI MmPRChetaI Ha PRChetaII Ha PRChetaII BtPRChetaII OoPRChetaII RnPRChetaII RnPRChetaII RnPRChetaII	VER MSREAVSCROLL HEF RELCCO ECCDINENT FER INVEST. C.C. PRORO TAMED FOR 6 V VFR MSREAVSCROLL HEF RELCCO ECCDINENT FER INVEST. C.C. PRORO TAMED FOR 6 V VFR MSREAVSCROLL HEF RELCCO ECCDINENT FER INVEST. C.C. PRORO TAMED FOR 6 V VFR MSREAVSCROLL HEF RELCCO ECCDINENT FER INVEST. C.C. PRORO TAMED FOR 6 V VFR MSREAVSCROLL HEF RELCCO ECCDINENT FER INVEST. C.C. PRORO TAMED FOR 6 V VFR MSREAVSCROLL HEF RELCCO ECCDINENT FER INVEST. C.C. PRORO TAMED FOR 6 V VFR MSREAVSCROLL HEF RELCCO ECCDINENT FER INVEST. C.C. PRORO TAMED FOR 6 V VFR MSREAVSCROLL HEF RELCCO ECCDINENT FER INVEST. C.C. PRORO TAMED FOR 6 V VFR MSREAVSCROLL HEF RELCCO ECCDINENT FER INVEST. C.C. PRORO TAMED FOR 6 V VFR MSREAVSCROLL HEF RELCCO ECCDINENT FER INVEST. C.C. PRORO TAMED FOR 6 V VFR MSREAVSCROLL HEF RELCCO ECCDINENT FER INVEST. C.C. PRORO TAMED FOR 6 V VFR MSREAVSCROLL HEF RELCCO ECCDINENT FER INVEST. C.C. PRORO TAMED FOR 6 V VFR MSREAVSCROLL HEF RELCCO ECCDINENT FER INVEST. C.C. PANDRO TAMED FOR 6 V VFR MSREAVSCROLL HEF RELCCO ECCDINENT FER INVEST. C.C. PANDRO TAMED FOR 6 V VFR MSREAVSCROLL HEF RELCCO ECCDINENT FER INVEST. C.C. PANDRO TAMED FOR 6 V VFR MSREAVSCROLL HEF RELCCO ECCDINENT FER INVEST. C.C. PANDRO TAMED FOR 6 V VFR MSREAVSCROLL HEF RELCCO ECCDINENT FER INVEST. C.C. PANDRO TAMED FOR 6 V VFR MSREAVSCROLL HEF RELCCO ECCDINENT FER INVEST. C.C. PANDRO TAMED FOR 6 V VFR MSREAVSCROLL HEF RELCCO ECCDINENT FER INVEST. C.C. PANDRO TAMED FOR 6 V VFR MSREAVSCROLL HEF RELCCO ECCDINENT FER INVEST. C.C. PANDRO TAMED FOR 6 V VFR MSREAVSCROLL HEF RELCCO ECCDINENT FER INVEST. C.C. PANDRO TAMED FOR 6 V VFR MSREAVSCROLL C.C. C.C. PANDRO TAMED FOR 6 V VFR MSREAVSCROLL C.C. C.C. PANDRO TAMED FOR 6 V VFR MSREAVSCROLL HEF RELCCO ECCDINENT FER INVEST. C.C. PANDRO TAMED FOR 6 V VFR MSREAVSCROLL HEF RELCCO ECCDINENT FER INVEST. C.C. PANDRO TAMED FOR 6 V VFR MSREAVSCROLL HEF RELCCO ECCDINENT FER INVEST. C.C. PANDRO TAMED FOR 6 V VFR MSREAVSCROLL REPREST. C.C. PANDRO TAMED FOR 6 V VFR MSREAVSCROLL REPREST. C.C. PANDRO TAMED FOR 6 V VFR MSREAVSCROLL REPREST. C.C. PAN	33 36 32 36 32 36 31 35 35 35 35 35 35 35

Molecular Cloning, Expression, and Enzymatic Analysis of Protein kinase C βl and βll from Inshore hagfish (*Eptatretus burgeri*)

	HXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX
EbPRCbetaI	MSEMDSEHLAREGETR-FARGGAVICRNVHEVRDHRFTARPFROPTCSHCKDFTWG GROGFOCVCSEVVHER 74
HsPKCbetaI	MADPAAGPPPSEGEZSTVR-EARRCALR RNVHEVRNHRFIARFFRQFTECSHCTDFIWG GRQGFQCVCCEVVHRR 77
BtPRCbetaI	MADPAAGPPPSEGEE-STVR-FAREGALRERNVHEVENHRFTARFFRQPTECSHCIDFIWGEGEQGFQCCVCFVVHER 77
DrPRChetaI	MTPSSDSDCFSTVR_FARKCALT KNVHEVRIHKFIAREPROFFCSHOIDFIWG GROEGCCVCCEVVHKR 7/
RnPKCbetaI	MADPAAGPPPSECE STVR-EARRCALR RNVHEVRNHRFTARFFRQPTFCSHCTDFIWGLGLQGFQSCVC
MmPRCbetaI	MADPAAGPPPSEGEE-STVR-FARRCALRCRNVHEVRNHRFTARFFRQFTFCSHCTDFIWG GRQGFQCVCCFVVHRR 77
HsPKCbetaII	MADPAGFPPSGSE_STVR-FARGALR RNVHEVRNHRF ARFFROPTICSBC DFIWG GROGFOC VCFVVHRR 77
BtPRCbetaII	MADPAAGPPPSEGEZSTVR-FARRCALR RNVHEVRNHRFLARFFRQFTFCSHCTDFIWG GRQGFQCVCCEVVHRR 77
OcPKCbetaII	MADPAAGOPPSEG2E-STUR-FARCALR RNVHEVRNHRFIARFFROFTCSHC DFING GROOFOCVCGEVVHRR 77
RnPKCbetaII	MADPAAGPPPS GD2 - STVR FARCALR RNVHEVRNBRF ARFFROPTICSBC DFIWG GROGOC VCCFVVHRR 70
MmPKCbetaII	MADPAAGPPPSEGEE-STVR-FARECALE RNVHEVENHEFTARFFROPTFCSHOTDFINGEGEOGCOVCEVVHER 77
	CXXXXXXC HXXXXXXXXXXXXXXXXXXXXXXXXXXXXX
EbPKCbetaI	CHEEVIECCECADEGAD TODERS THATE HTYS FTECHCGS LYGL HOGMEOT CMNVHK CONVESICE/DHTE 15-
HsPKCbetaI	CHEFVIFSCPCADRGPASDDPRSRHKFKIHTYSSPTFCDHCGSLLYGLIHQCMRCD1C3MNVHKCCMNVPSLCGTDHTE 15
BtPRCbetaI	CHEEVIFSCFCADRGPASDDFRSRHSFK HTYSSFTFCDHCGSLLYGLIHQGMRCPTCMMNVHKSCVMNVPSLCGTDHTE 15
DrPRCbetaI	CHEFVIFSCPC ADAGP SDDPRSHIPS HITS SPIFCDICGSLIGLI HOGMCCI CIMN THROUGH VPSLCGIDHTE 15 CHEFVIFSCPC GDRGP SDDPRSHIPS FR HTY SPIFCDICGSLIGLI HOGMCCI CIMN THROUGH FR VPSLCGIDHTE 15
RnPKCbetaI	CHEFVIFSCECADRGPASDDFRSRHAFKTHTYSSFFFCDHCGSLLYGLIHCCMRCDTCSMNVHKSCVANVPSLCGTDHTE 15
MmPRCbetaI	CHEEVITESCPCADRGPASDDFRSEHEFK HTYSSFFFCDHCGSLLYGLIHQCMRCDTCMNVHK2CVMVVFSLCGTDHTE
HsPKCbetaII	CHEFV FSCFCADRGPASDDFRSRH5RFTHTYSSFTFCDHCGSLLYGLH6CMRCD1C3MNVHRCCVMVVPSLCGDHTE 15
BtPRCbetaII	CHEFVIFSCFCADRGPASDDFRSRHAFKTHTYSSFFFCDHCGSLLYGLIHQCMRCDTCSMNVHRCCVMNVFSLCGTDHTE 15
OcPRCbetaII	CHEFVITSCFCADRGPASDDFRSRHSFKIHTYSSFTFCDHCGSLLYGLTHQGMRCDTCMMNVHKCVMNVPSLCGTDHTE 15
RnPKCbetaII	CHEFVIFSCFCADRGPASDDFRSRHSFKIHTYSSFTFCDHCGSLLYGLTHCCMRCDTCSMNVHKSCMNVFSLCGTDHTE 15
MmPRCbetaII	CHEEVIFSCFCADRGPASDDFRSRHSFKIHTYSSFTFCDHCGSLLYGLIHQGMBCDTCAMNVHRCOVAVFSLCGTDHTE 15
EbPKCbetaI	REGELTINA MIGPHTL WITH REARNED EMPENDED SOFTWIRE UPDER DES RORTET IR AT INP. W. P. P. P. P. I. R. B. P.
HsPKCbetaI	REGRIVICA TOR-DVLIVIVELARNIVEMDENGLSDEYVELEL POPESESEGETETIE SUNP WEIFEFELES DK 230
BtPKCbetaI	REGENTICA ER-EVLIV VREARNIVEMDENGLSDEVVRLRIFDERSESRORTRTIK SLNP WEIFEF
DrPKCbetaI	RRGR HFAATTG-NUL V VRAANLVENDPSGLSDFYVRLKLEPDFRESSRORTHTIRGSCNPW ELFEFELKS DK 23
RnPKCbetaI	RRGRIVICA DR-EVLIV VRLARNLVEMDPAGLSDPYVRLRLIPDPRSESRORTRTIK SLNPEMBERFRE KESDR 230
EbPKCbetaI	REGRET LIGHT DR. DR. DR. ANNI VERDERGESDEVER RUDDERSESRORTRETIK SUNDAR EFSE LIGSDR 23
HsPKCbetaII	REGET VICA TDR-DVL V VR. ARNIVENDENGLSDFYVRIRL FDFRSESRORTKTIK SLNF W EIFSFLK SDK 23
BtPKCbetaII	RRGRTVICA TER-EVLIV VRIARNIVEMDENGLSDEVVRLRLIPDPRSESRORTRTIK SLNPEW BEFREFLKeSDK 23
DrPKCbetaII	REGREAT OF A PARTY AND A RELEVANT AND A REAL AND A
RnPKCbetaII	REGRIVICA TOR-EVILV VELARNIVEMDENGLSDPYVELEL POPESESEQETETIC SUNP WE PRE LK SDE 230
MmPKCbetaII	REGRIVICA DR-EVLIV.VREARNIVEMPENDENGLSDEVVRLELIEDERSESKORTRIIKESLEEWNETERFOLKSSDK 230
EbPRCbetaI	DRRLSTEVWEWDRTTRNDFMGSMSFCVSELVKCQ/DCWYRLLSCEEGSVYNVEVAFECEES-LELRORIORSTSSS 31
BtPRCbetaI	DRRLSVEIWEWDLTSRNDENGSLSFGISELORAGYDCWFRILSCEEGEVENVEVPERGSEINBELROR EFARIGPGPRT 31
OcPRCbetaI	DRRLSVEIWEWDLISRNDEMGSISEGISELOKAGUDCWERLLSCEEGEVENVEV 2E23SE3NEELEGKIEFARIDQGTKT 31
RnPKCbetaI	DRRLEVEYWEWDROFRNDINGSNSFOISELDROSVDEWERLIAGEEGMERNVEVEVED 22 BINRELSRREPART 300 TRN 31. DRRLEVEIWEWDLTSRNDINGSNSFOISELDRAGVDCWERLIASEEGYENVEVENBESENRELSRREPART 300 TRA 31
MmPKCbetaI	DRRLEVEIWEWDLTSRNDFMGSISFGISELORAGVDEWERLISCEEGEVENVEVPFS3SE3NEELRORSEFARI30GTRA 31
EbPRCbetaII	DRRLS E WEWDRTTRND MGS SPC SEL VRCO DGW RT LSDEEGE YN VEVAL DE EALER ARLORS OT DRSSR 31. DRRLS E WEWDLTSRND MGS SPC SEL DRSSR VDCW RT LSDEEGE YN VEVAL DE EALERAND FRANK
BtPRCbetaII	DRRLEVEIWEWDLTSRNDEMGSLSFGISELOKAGYDCWERLLSCEEGEYENVEVPFEGSEBINEELROKTEFARIOPOPRT 31
OcPRCbetaII	DRRLEVEIWEWDLISENDIMGSLSPGISELOKAGVDGWRRLLSEBEGENVEVEPISESEDNEELROKEFARTIGGETKT 31 DRRLEVEIWEWDLISENDIMGSLSPGISELOKAGVDGWRRLLSEBEGENVEVEPISEDERENDELROKEFARTIGGETKT 31
RnPKCbetaII	DRRL VEIWEWDLISRNDEMGSLSFGISELCKAGYDCWERLLSCEEGEVENVEV 25232 INEELROK EFARI 200TKA 31
MmPRCbetaII	DRRLEVELWEWDLTSRNDEMGSISFGISELOKAGVDEWERILSCEEGEVENVEVPEGSSE3NEELROKPERART3QGTKA 31
	CYCYYC
	GAGAAG
EbPRCbetaI	ROSPAENOFSESRSLSGLDEVRLEDFIFLIVLGROSSGRVNLAEORVITCOLVALRILRREVVIQDDEVECTNVEREVLAL 39
BtPKCbetaI	PERTING ISK DUNGURDERKEIDEFFE VLORGSFGRVMLSERR ^D TDELVAVRILKREVVIQDEVECTVVERFVLAL 35
OcPKCbetaI	PEEKTINTISK DNNGNRDFWRLIDFAFL VLGRGSFGRVMLSERKOTDELYAVRILRREVVIQDDEVECINVERFVLAL 39
DrPKCbetaI	TDGASSROLSK DANGNODENKLADEN FIN VLGRGSPGRVMLABRROSDELVAVRILKKEVVIQDEVECTVVERFVLAL 39
MmPKCbetaI	PERTANTISK DNNGNRDERKLIDE FLYLGRGSFGRVMLSERKUIDELFAKLIKKUVIQDD VECIVVERVIAL 39
EbPKCbetaII	ROSPAENOESESRSLSGLDEVELEDETEL VLGRGSEGEVELAEOR TOOLYAIRILREVVIQDDEVECTEVEREVLAL 39
HsPKCbetaII	PEERTINTYSK DNNGNRDFMRLTDFYFLYULGRGSFGRVMLSERR <mark>OTD</mark> ELYAVRILRRCVVIQDDEVECTMVEREVIAL 39
OcPKCbetaII	PERKINT ASA PANGANGANGANGANGANGANGANGANGANGANGANGANGA
DrPKCbetaII	TDGSSSNAISK DSNGNRDFMRLSDFNFL VLGRGSFGFVMLAERKDADELFAIRILRREVVIQDDEVECTMVERFVLAL 39
RnPRCbetaII	PEEKTANTISK DNNGNEDEKRL DE FLVLGRGSEGEVMLSERK TDELVAVRILREVVIQDEVECTVVERFVLAL
MMPRCDetaII	PERKTANTI IST PNNGNRDINKLIDE FIL VLOKOSFORVMISERKETDELI AVRILKREVVIQDDE VECTIVER VLAL 39
EbPRCbetaI	SNRFFFLT%LHSCFQT*DRLYFVMEFVNGGDLMYTIQQVRFFREPA*VFYA*BLAVGLTVLaGRGTIYRDLRLDNVMLDS 47:
BtPRCbetaI	PORPETENT INSCROMENTING AND A COMPANY AND A
OcPKCbetaI	PCRPFFLTCLHSCFCTTDRLYFVMEYVNGGDLMY IQQVCRFREPH VFYA BIAIGLFLOSKGTIYRDLKLDNVMLDS 47
DrPRCbetaI	SCREPFLT LHSCFQTYDRLYFVMEYISGGDIME IQQTCRFREPL VFYA EIAIGLEL SRGITYRDLRLDNVMLDS 47.
RnPRCbetaI	PCRPFLTILHSCFQTYDRLYEVMEYVNGGDLMY IQQVCRFREPS/VFYAPEIAIGLFL2SKGTIYRDLRLDNVMLDS 47
MmPKCbetaI	PORPPETTURSCROTMORLYFVMEYVNGGDIWY IQQVGRFREPH VFYA EIAIGL FLOSRGTIYRDLRLDRVMLDS 47
HsPKCbetaII	PORPETITILISCECTORI VENE VIGOUNT AND TROUGHT POR TAIGUET LOS RECEIRED NUMBER 47
BtPKCbetaII	PORPFELT LHSCFQTYDRLYFVMEXVNGGDLMY IQQVGRFREPAVFYA EIAIGLELDSRGIIYRDLRLDNVMLDS 47
OcPRCbetaII	PCRPFLTCLHSCFQTODRLYFVMEYVNGGDIMY IQQVCRFREPH VFYA: EIAIGLEFLQSRGIIYRDLRLDNVMLDS 470
DrPKCbetaII	SCREEFLTLHSCECT DRUFTINGGDINY IQQVCEFREEH VFYALEIATGL FLHSRCVIYRDLRLDNVMLDA 47
MmPRCbetaII	PREPETED INSCRUMENTER VAN VAN GODIANT I COMPRESSION VEVALED ALGE DE DESKELLVRULELDNVMLDS 47
EbPRCbetaI	EGHIRIADFEMCRENV/201TTRTFCGTPDY1APEIIAYQPYGGSVENNAYGVLLYEMIAGE PFDGEDEDELFQSIMEH 55
HsPKCbetaI	EGHIRIADFCMCKENT OF TTTTTFCGTFDYIAFEIIAYQFYGRSVCWWARCVLLYEMIAGO FFEGEDEDELFQSIMEH BCHIRIADFCMCKENT OF TTTTTFCGTFDYIAFEIIAYQFYGRSVCWWARCVLLYEMIAGO FFEGEDEDELFQSIMEH 55
OcPEChetaI	BORTKTADFORCRENT OF WITTFECOTEDY LAPELTAXOPYORSVEWWARGVLLVENLAGO PERGEDEDELFQSIMEH 55
DrPRCbetaI	BGHIRIADFCMCRENM DCC TTTTFCGTFDYIAPBIIAYQPYGRSVCMAAYCVLLYFMLAGC FFDGEDEDELFQSIMEH 55.
RnPKCbetaI	EGHIRIADFEMERENTADTVTTRTFEGTFDYIAPEIIAYQPYGRSVEWAAFEVLLYEMLAGQ PFEGEDEDELFQSIMEH 55
MmPRCbetaI	EGHIRIADFCMCRENIND TRIFFCGTFDYIAFEIIAYQFYGRSVChharcvllyemlago ffegededelfqsimeh 55
EDPRCbetaII	EGHTKTADFCMCRENVN33TTTTFFGGFEDYTAPETTAYGPYGGSVCWWAYCVLLVEMLAGE FFDGEDEDELFQSIMEH 55
BtPKCbetaII	BGHIRIADFGMCRENT DEVTIRE FCGTPDTIAPETIATOPTCRSVEWAACVLITERTAGE PFEGEDEDELFQSIMEH 55
OcPRCbetaII	EGHIRIADFCMCRENI D TTRTFCGTFDYIAPEIIAYQFYGRSVCMMAFCVLLYEMLAGO PFRGEDELFQSIMEH 55
DrPKCbetaII	EGHIRIADFEMERENMIDEVTTRTFEGTFDYIAPEIIAYQFYGRSVENNAFEVILYEMIAGQEPFDGEDEDELFQSIMEH 55
RnPKCbetaII	EGHIRIADFCMCRENTAD VTRTFCGTPDYIAPEIIAYQFYQRSVCMAAFCVLLYEMLAGO FFEGEDEDELFQSIMEH 55
LIETOCOCCUL	

EbPRCbetaI NVEYPE	MSREAISVCRGLL	RHPRRLGCC	EGEODIREH	FFR ICWERL	ECREIQPPERPK	RORODAGNEDRA	TT 633
HsPKCbetaI NV YPR	MSREAVAICRGIM	RHPGRRLGCG	EGERDIREH	FFRICKERL	E <mark>r</mark> reigppyrpk	RDKRDTSNEDKE	TR 636
BtPKCbetaI NV YPK	MSREAVAICRGLM	RHPRRLGCC	EGERDIREH	FFRIICWERL	E <mark>R</mark> REIQPPYRPK	RDKRDTSNEDKEF	TR 636
OcPKCbetaI NV YPR	MSREAVAICRGLM	RHPGRRLGCC	EGERDIRDH	FFRICKERL	B <mark>R</mark> REIQPPYRPK	RDKRDTSNEDKE	TR 636
DrPRCbetaI EVSYPE	MSREAVAICRGLM	RHPCRRLGCC	LEGERDIREH	FFR VCWERL	ONRE AÖBBAKEK	KNRHDTCNFDR 2F	TK 632
RnPKCbetaI NV YPK	MSREAVAICRGIM	RHPCRRLGCC	EGERDIREH	FFRICWERL	B <mark>RREIQPPYRPK</mark>	RDKRDTSNEDKOF	TR 636
MmPRCbetaI NV YPR	MSREAVAICRGLM	RHPCRRLGCC	EGERDIREH	FFRIICWERL	e <mark>r</mark> reigppyrpk	RDKRDTSNEDKEF	TR 636
EbPKCbetaII NVEYPR	MSREAISVCRGLL	RHPERRLGCC	EGECTIREH	FFR ICWBRL	E<mark>CREIQPPFRPK</mark>	CGR-DAVNEDKCE	TR 631
HsPKCbetaII NV YPK	MSREAVAICRGLM	RHPCRRLGCC	EGERDIREH	FFRIICWERL	BREIGFFYRFR	CG-RNAENEDREE	TR 635
BtPKCbetaII NV YPK	MSREAVAICRGLI	RHPRRLGCC	EGERDIREH	FFRICKERL	BREIQFFYRFR	CG-RNAENFDRFF	TR 635
OcPKCbetaII NV YPK	MSREAVAI CRGLM	RHPGRRLGCG	EGERDIRDH	FFRIICWERL	BREIQPFYRFK	CG-RNAENEDREE	TR 635
DrPRCbetaII EVSYPR	MSREAVAICRGLM	RHPCRRLGCC	EGERDIREH	FFRENCWERL	BEREVQPPERER	CG-RDAENEDREE	TR 632
RnPKCbetaII NV YFR	MSREAVAICRGLM	RHPCRRLGCG	EGERDIREH	FFRICKERL	B BREIQPFYRPK	CG-RNAENEDREE	TR 635
MmPRCbetaII	MSREAVAICRGLM	RHPCRRLGCC	EGERDIREH	FFRICWERL	BREIQPFYRFK	CG-RNAENEDREE	TR 635
EbPRCbetaI	DSVIET	PVDRLI	VLTLDC	SDFAGE	SETNPEP	IIHV	668
HsPKCbetaI	QPVEL T	PTDKLE	IMNLDQ	NEF GF	SYTNPER	VINV	671
BtPRCbetaI	QPVEL 7	PTDKLI	IMNLDC	NEF GE	SYTNPER	VINV	671
OcPKCbetaI	QPVE 1	PTDKL	IMNLDQ	NEF GF	SYTNPER	VINV	671
DrPKCbetaI	MAVELT	PTDKLI	IMNLDC	NEFOGE	SYDNPER	VIOV	667
RnPKCbetaI	OPVELT	PTDKLE	IMNLDC	NEFAGE	SXINPER	VINV	671
MmPKCbetaI	OPVELT	PTDKLI	IMNLDC	NEFAGE	SXINPER	VINV	671
EbPKCbetaI	I VOPVILT	PEDEY	INNIDC	SEFCGE	SYMNPAP	GPSL	666
HsPKCbetal	I HPPVIT	PEDOEN	RNDO	SEFEGE	SEVINSEE	LKPEVKS	673
BtPRCbetal	I HPPVIT	PEDOES	RNIDC	SEFEGE	SEMISEP	LEPEVES	673
OcPECbetaT	T HPPVIT	PPDOES	I BIN DO	SEFEGE	SEMINSPE	LEPEVES	673
DrPKChetaI	T HPPVI	PEDOES		DEFECF	SETNER	PAMEAOS	670
Paperchetal	T HPPM	PEDORI		SEFECE		I KPEVKS	673
Mapperchatal	T HDDD	DEDORT				I POPULES	673

[Fig. 3] Multiple amino acids sequence alignment analysis of PKC subtypes in various species.

The GenBank accession numbers used in the alignment are shown in table 2. Identical amino acid residues are darkly shaded, similar amino acids are lightly shaded, unrelated residues have a white background, and amino acid numbers are shown on the right. "HX₁ $_2$ CX₂ CX₁ $_3$ CX₂ CX₄ HX $_2$ C₇C" in the first square box is C1 motif, that exists in both C1A and C1B. The two circles above the sequence show the Asp residues binding to calcium. The second square box shows the ATP binding site, "GXGXXG". The first inverted triangle is invariant Lys and the second inverted triangle is Met as a gatekeeper residue.

(Isopropyl- β -D-thiogalactopyranoside) added to it to a final concentration of 1 mM and grown at 20°C about 24 h. The induction of the target proteins was checked by SDS-PAGE (10% running gel, 5% stacking gel) and Western blotting. In order to obtain target proteins (*EbPKC* βI and βII) in large scale, the cells were inoculated in LB broth (500 mL) and grown at 37°C until the OD₆₀₀=0.6.

5. SDS-PAGE and Western blot

For the electrophoresis procedures, all samples were denatured in buffer containing 60mM Tris/pH 6.8. 25% glycerol, 2% SDS. 14.4mM 2-mercaptoethanol, and 0.1% bromophenol blue, then boiled for 5min. Purified $EbPKC\beta I$ and βII were separated by 10% SDS-PAGE (Bio-Rad, USA). Prestained molecular weight markers (Bio-Rad, USA) were run as standards on each gel. Following electrophoresis, the gels were stained with Coomassie brilliant blue R-250.

Western blotting was performed using rabbit polyclonal anti-His antibody (1:2000, Santa Cruz Biotechnology) and rabbit monoclonal anti-His antibody (1:1000, Santa Cruz Biotechnology). Prestained molecular weight markers (Bio-Rad, USA) were run as standards. The electrophoresed samples transferred nitrocellulose were to membranes (Schleicher & Schuell. Co., USA) using a Hoefer transblotting system (Pharmacia. Co., USA). Following this transfer, the membrane was blocked with 3% BSA in TPBS [200 mM Tris (pH 7.0), 1.37 M NaCl, 1% Tween-20] for 30 min at room temperature. Primary antibody was attached to the target proteins at 4° for 16 h. Secondary antibody was attached to the target proteins at 4° C for 1.5 h.

6. Activity assay

Phosphorylation by PKC of its specific substrate alters the peptide's net charge from +1 to -1. PepTag® Non-Radioactive PKC Assay (Promega) was used to analyze the activities of $EbPKC \beta I$ and EbPKC \$II. For qualitative analysis, proteins were diluted to 1, 2, 5, and 10 ng/ μ l, and reacted with substrate at 30°C for 30 min. After reaction, samples underwent electrophoresis on gel, which was made of 50 mM Tris-HCl(pH 8.0) and 0.8% agarose, over 20 min. Phosphorylated peptide was separated for quantitative analysis. At 95°C, it was completely melted and Gel Solubilization Solution, glacial acetic acid, and distilled water were added. Using a spectrophotometer, we assessed the absorbance at A₆₅₀ and calculated activity using the following equation:

 $A = \epsilon BC$,

where: A = absorbance of the sample, ε = the molar absorptivity of the peptide in L/mol • cm⁻¹, B = the width of the light cell, and C = the concentration of the peptide in mol/L of the sample read.

III. Results

1. Cloning and sequence analyses of *EbPKC* β and β \parallel

In order to identify the partial sequences of $EbPKC\beta$, databases of other PKCs were obtained using NCBI sequence data. These sequences were used to design degenerated primers. The initial partial sequences were obtained through PCR amplification of inshore hagfish cDNA, including the brain, tentacle, gill, root of the tongue, spinal cord, heart, liver, gut, muscle, skin, and mucous gland. In order to isolate full-length inshore hagfish PKC β I and β II, the partial sequences were used as bases for gene-specific primers for RACE PCR.

As a result, the full nucleotide sequences of *EbPKC* βI and *EbPKC* βII were 2,499 bp and 2,658 bp, respectively. The $EbPKC\beta I$ sequence was composed of a 238 bp 5'-untranslated region (5'-UTR), a 2,007 bp coding region, and a 254 bp 3'-untranslated region (3'-UTR) [Fig. 1]. 3'-UTR of EbPKC \$\beta I had a miR-199-5p binding site from 73 to 78 (5'-TACTGG-3'). The EbPKC \$\mathcal{B}II sequence was composed of a 235 bp 5'-untranslated region (5'-UTR), a 2,004 bp coding region, and a 419 bp 3'-untranslated region (3'-UTR) [Fig. 2]. 3'-UTR of EbPKC \$\beta II had a miR-203a-5p binding site from 328 to 334 (5'-GATCCAT-3'). The EbPKC \$I codes 668 amino acids, which the molecular weight is approximately 76.43 kDa, and the EbPKC BII codes 667 amino acids, which the molecular weight is approximately 76.08 kDa. These sequences were submitted to the NCBI database $[PKC \beta]$ I(**MH350863**), PKC β II(**MH350864**)].

EbPKC βI and *EbPKC* βII have the same sequences in conserved regions (C1, C2, C3, and C4 domain), but not in the C-terminal called the V5 domain. C1 domains were highly conserved DAG/PMA binding sites with a characteristic HX₁ $_2$ CX₂ CXnCX₂ CX₄ HX₂ CX₇C motif (H, histidine; C, cysteine; X, any other amino acid; n is 13). C2 domains contained a calcium binding loop, which has several highly conserved Asp residues, and a RACK (receptor for activated C-kinase) binding site.

It was confirmed that both *EbPKC* βI and βII had these highly conserved motifs. Kinase domains include an ATP-binding site (GXGXXG; G is glycine and X is any other amino acid), invariant Lys, and gatekeeper residue. cPKCs and nPKCs

have Met as a gatekeeper residue, while aPKCs use Ile. It was confirmed that *EbPKC* βI and βII have these residues as well. In the case of V5 domains, which are about 50 residues of C-terminus, *EbPKC* βI and βII differ significantly. Thus, βI and βII were determined by referencing previous studies [Fig. 3].

2. Phylogenetic tree of *EbPKC*_{\$\mathcal{B}} | and \$\mathcal{B}\$ ||

In order to determine the evolutionary relationship of *EbPKC* βI and βII with other families of the PKC, a phylogenetic tree was constructed.

Phylogenetic analysis was performed with the amino acid sequences of human PKCs and the PKCs of other species obtained from GenBank using neighbor-joining methods [Fig. 4]. Based on a comprehensive phylogenetic analysis, cPKCs, nPKCs, and aPKCs were classified. In other species, βI was more closed compared to the βI of another species but not in the case of fish (*DrPKC* βI and *EbPKC\beta I*).

3. Tissue distribution of *EbPKC_B1* and *Bll* by qRT-PCR analysis

The distributions of *EbPKC* βI and βII transcripts in different organs were examined by RT-PCR. The results of qPCR indicated that *EbPKC* βI and βII were expressed in different organs, including the brain, tentacle, gill, root of tongue, spinal cord, heart, liver, gut, muscle, skin, and mucous gland. The expression pattern of *EbPKC* βI was found at its highest levels in the root of the tongue and spinal cord [Fig. 5A]. The expression pattern of *EbPKC* βII was found at high levels in the gill, liver, and gut [Fig. 5B].



[Fig. 4] Phylogenetic relationship of $EbPKC \beta I$ and βII with other PKC subtypes. In this neighbor-joining phylogram, all individuals are represented and the branches are based on the number of inferred substitutions, as indicated by the bar. The square indicates $EbPKC \beta I$ and $EbPKC \beta II$.

Molecular Cloning, Expression, and Enzymatic Analysis of Protein kinase C βl and βll from Inshore hagfish (*Eptatretus burgeri*)



[Fig. 5] Tissue-specific distribution of *EbPKC* βI and βII Quantitative real-time PCR of *EbPKC* βI and *EbPKC* βII in various tissues. Mean of mRNA levels in *E. burgeri* tissues were analyzed by real-time PCR, and $2 - \Delta \Delta Ct$ levels were calculated relative to the tissue with the lowest expression (PKC βI from gut tissue and PKC βII from muscle tissue) set to 1 and normalized against 18s-rRNA expression. Each experiment was done in triplicate.



[Fig. 6] SDS-PAGE and Western blot analysis of EbPKC \$\mathcal{P}I\$ and \$\mathcal{P}II\$.

10% SDS-PAGE gel and coomassie R-250 blue was used to perform SDS-PAGE. Anti-His antibody was used for Western blotting. Predicted recombinant *EbPKC* β s Molecular weight is approximately 92 kDa, as they include Trx-tag, S-tag, His-tag, and other amino acids. M, standard size marker; N, cell lysate from IPTG-not induced *EbPKC* β -expressing *E. coli* strain BL21 (DE3); I, cell lysate from 1 mM IPTG-induced *EbPKC* β -expressing E. coli strain BL21 (DE3).

4. SDS-PAGE and western blot

In order to select recombinants of $EbPKC\beta I$ and

 βII , transformed cells were grown in LB broth (5 ml) containing ampicillin (100 µg/ml) and had IPTG added to a final concentration of 1 mM. The

sample was checked with induction of target proteins by SDS-PAGE (10% running gel, 5% stacking gel) and Western blotting was performed [Fig. 6A, 6B].

Although the size of *EbPKC* βI and βII is approximately 76 kDa in both cases, the target size on SDS-PAGE is about 92 kDa, because pET32b vector expresses other proteins, including Trx tag, S tag, and His tag. Recombinant *EbPKC* βI and βII were purified by affinity column with nickel resin at 4°C. SDS-PAGE was onducted with 10% acrylamide gel [Fig. 7A, 7B]. Purified samples were dialyzed in order to check the *EbPKC* β 's activities.



- [Fig. 7] SDS-PAGE of *EbPKC J1* and *J11* purified by affinity chromatography.
 10% SDS-PAGE gel and coomassie R-250
 - blue was used to perform SDS-PAGE. Predicted recombinant EbPKC β s Molecular weight is approximately 92 kDa. M, standard size marker; -10, sample obtained for 10 minutes after passing through an elution buffer; 1~10, sample obtained for the next minute; +10, sample obtained during the last 10 minutes.

5. Activity assay

In order to check whether recombinant $EbPKC\beta I$ and βII proteins have PKC activity, we conducted activity assay using substrate which could be phosphorylated by PKC. Consequently, we identified that they can indeed phosphorylate substrate [Fig. 8].



[Fig. 8] Qualitative analysis of $EbPKC \beta I$ and EbPKC \$II. Substrate was phosphorylated by purified proteins at 1, 2, 5, and 10 ng/ μ l, respectively. Lane 1 indicates negative control and lanes 2-5 indicate reactants which contain substrate and diversely diluted purified protein (1, 2, 5, and 10 parts $ng/\mu l$). The upper are phosphorylated substrate and the lower parts are non-phosphorylated substrate.

The results of quantitative analysis showed that in the case of *EbPKC\betaI*, it phosphorylated substrate at 14.52, 22.36, 29.25, and 40.43 pmol/min at 1, 2, 5, and 10 ng/µl, respectively [Fig. 9A]. *EbPKC\betaII* phosphorylated substrate at 0.86, 1.27, 10.32, and 43.87 pmol/mine at 1, 2, 5, and 10 ng/µl, respectively [Fig. 9B].



Molecular Cloning, Expression, and Enzymatic Analysis of Protein kinase C βl and βll from Inshore hagfish (*Eptatretus burgeri*)

[Fig.	9]	Quantitative <i>EbPKC &II</i> .	analysis	of	EbPKC \$I	and
		We checked	the amou	unt o	f phosphory	lated
		substrate ti	reated w	ith	EbPKC \$I	and

substrate treated with *EbPKC \beta I* and *EbPKC \beta II* 1, 2, 5, and 10 ng/ μI . (A) Activity of *EbPKC \beta I*, (B) Activity of *EbPKC \beta II*.

4. Discussions

In this study, we identified sequences of *EbPKC* βI and *EbPKC\beta II*, which originated from one PKC β gene, and conducted enzymatic analysis. As a result, *EbPKC\beta II* and *EbPKC\beta II* were found to encode 668 and 667 amino acids, respectively. They also showed PKC activity.

equence for comparison	Species	NCBI accession number
HsPKCa		BAU98542.1
HsPKCβI	Homo sapiens	NP_997700.1
HsPKCβII	(Human)	NP 002729.2
HsPKCô		NP_001303256.1
HsPKCe		NP 005391.1
MmPKCa		AAA39934.1
MmPKCβI		NP_032881.1
MmPKCβII	Mus musculus	NP_001303601.1
MmPKCőI	(House mouse)	AAF79208.1
MmPKCõII		NP_035233.1
MmPKCλ		BAA32499.1
МаРКСВІІ	Monopterus albus	XP 020453442.1
MaPKCBI	(Asian Swamp Eel)	XP 020453441.1
MaPKCe	and a second and a second Review of the	XP 020460965.1
T h DY CO	Labrus bergylta	XD 020502505 1
соркср	(Ballan wrasse)	XP_020502596.1
AmPKCβI	Astyanax mexicanus	XP 022530628.1
AmPKCβII	(Mexican tetra)	XP 022530629.1
AmPKCŋ		XP 022540784.1
ВрРКСβ	Boleophthalmus pectinirostris	XP 020775613.1
BpPKCŋ	(Boleophthalmus)	XP 020775049.1
SdPKCβ	Seriola dumerili	XP_022612562.1
SdPKCõ	(Greater amberjack)	XP 022600899.1
SdPKCŋ		XP 022598245.1
NfPKCa	Nothobranchius furzeri	SBS59970.1
NfPKCy	(Turquoise killifish)	SBP44224.1
AsPKCa	Aphyosemion striatum	SBP12178.1
AsPKCy	(killifish)	SBP15911.1
ApPKCõ	Acanthochromis polyacanthus	XP 022046770.1
ApPKCe	(Spiny Chromis)	XP 022045543.1
ApPKCn		XP 022077382.1
FhPKCô	Fundulus heteroclitus	XP 021172777.1
1962-2 2005	(Mummichog)	1963.5 0 74 (1996-563)1161
OIPKCö	Oryzias latipes	XP 011472964.1
((((((((((((((((((((((((((((((((((((((((Japanese rice fish)	and the second s
OmPKCe	Oncorhynchus mykiss	XP_021432053.1
OmPKCŋ	(Rainbow trout)	XP_021440637.1
NkPKCa	Nothobranchius korthausae	SBO50761 1
- 1000 AD 21 10	(killifish)	
NrPKCa	Nothobranchius rachovii	SBR 80037 1
MEROU	(killifish)	351(09057.1
DrPKCβI		XP_005170901.1
DrPKCβII	Danio rerio	NP_957272.1
DrPKCλ	(Zebrafish)	AAK91291.1
DrPKCô		NP_999873.1
DrPK Cn		NP 001038271 1

<Table 2> Sequences used in this study

In order to check tissue-specific expression, we conducted qPCR. *EbPKC* βI was highly expressed in the root of the tongue and spinal cord, and *EbPKC*

 βII was highly expressed in the gill, liver, and gut. In particular, a clearly large amount of mRNA was transcribed in the root of the tongue. In several species, PKCB is involved in the immune system, signaling, such as in immunoreceptor immunodeficiency. and the development and activation of B cells (Kawakami et al., 2002). Therefore, we predict that the root of tongue can act as not only a predatory organ but also a sensory or immune-related organ through contact with the environment. In the jawed vertebrate, PKC β1 is highly expressed in the brain (Goldberg and Steinberg, 1996; Ohno et al., 1987). However, in hagfish (the jawless vertebrate), PKCB1 is highly expressed in the spinal cord but not in the brain. It is suggested that the spinal cord of the hagfish is more important than that of a jawed vertebrates in the role of the central nerve system.

In vertebrates, PKC β I and PKC β II are regulated by miR-203 and miR-7, respectively. Hagfish has miR-199 gene and miR-203a gene (Heimberg et al., 2010). In this study, *EbPKC\betaI* and β II contained miR-199 and miR-203a binding sites in their respective 3'-UTR. Therefore, it is possible that Ebu-miR-199 and Ebu-miR-203a regulate *EbPKC\betaI* and *EbPKC\betaII*, respectively.

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