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Epithelium specific ETS transcription factor, ESE-3, of *Protobothrops flavoviridis* snake venom gland transactivates the promoters of venom phospholipase A_2 isozyme genes

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ABSTRACT

Protobothrops flavoviridis (habu) (Crotalinae, Viperidae) is a Japanese venomous snake, and its venom contains the enzymes with a variety of physiological activities. The phospholipases A_2 (PLA_2s) are the major components and exert various toxic effects. They are expressed abundantly in the venom gland. It is thought that the venom gland-specific transcription factors play a key role for activation of PLA_2 genes specifically expressed in the venom gland. Thus, the full-length cDNA library for *P. flavoviridis* venom gland after milking of the venom was made to explore the transcription factors therein. As a result, three cDNAs encoding epithelium-specific ETS transcription factors (ESE)-1, -2, and -3 were obtained. Among them, ESE-3 was specifically expressed in the venom gland and activated the proximal promoters of venom PLA_2 genes, which are possibly regarded as the representatives of the venom gland-specific protein genes in *P. flavoviridis*. Interestingly, the binding specificity of ESE-3 to the ETS binding motif located near TATA box is well correlated with transcriptional activities for the venom PLA_2 genes. This is the first report that venom gland-specific transcription factor could actually activate the promoters of the venom protein genes.

1. Introduction

The venom gland of Viperidae snakes, which is an oral exocrine gland relating to salivary gland, has the capacity to secrete a rich source of biologically and physiologically active proteins. In the past, much toxinological researches have been done for a vast amount of the knowledge, such as the structure and function relationships of venom proteins (Kini and Doley, 2010; Shimohigashi et al., 1995), the molecular evolution of venom isozymes (Nakashima et al., 1993, 1995), the specified clinical issues (Gutiérrez et al., 2009), *etc*. However, the transcriptional mechanisms for expression of venom protein genes are poorly understood, due mainly to the absence of established venom gland secretory cell lines. This is the first report regarding to the

Abbreviations: PLA_2, phospholipase A_2; *P, protobothrops*; ETS, E twenty-six; ESE, epithelium-specific ETS transcription factor; CHO, Chinese hamster ovary; FITC, fluorescein isothiocyanate; EST, expressed sequence tag; IPTG, isopropyl-β-D-thiogalactopyranoside; EMSA, electrophoretic mobility shift assay; ds, double-stranded; TSS, transcription start site.

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transcription factor indispensable to venom gland-specific gene expression for venomous snakes.

Protobothrops flavoviridis is a Japanese venomous snake inhabiting the southwestern islands of Japan. Its venom is a complex mixture of proteins such as phospholipases A_2 (PLA_2s) (Oda et al., 1990; Kihara et al., 1992; Chijiwa et al., 2003; Yamaguchi et al., 2001; Yoshizumi et al., 1990; Liu et al., 1990; Murakami et al., 2009), metalloproteases (Takahashi and Osaka, 1970; Miyata et al., 1989), serine proteases (Deshimaru et al., 1996), C-type lectins (Atoda and Morita, 1993), vascular endothelial growth factor (VEGF) (Yamazaki et al., 2003), *etc*. PLA_2s in particular are the major components of the venom and expressed only in the venom gland.

PLA_2s catalyze the hydrolysis of glycerophospholipids with a requirement for Ca^{2+}. Animals contain a variety of secreted PLA_2s as toxic components, digestive (Verheij et al., 1981) and non-digestive enzymes (Dijkstra et al., 1981; Kramer et al., 1989). These PLA_2s are classified into several groups based on sequences, molecular weights, disulfide bonding patterns and so on (Kini, 1997). The venom PLA_2s are classified into group II, whereas pancreatic PLA_2s belong to group I (Kini, 1997). Six PLA_2s from *P. flavoviridis* venom are divided into two subgroups according to the kind of amino acid at position 49 (Maraganore et al., 1984). The three isozymes, PLA2 (Oda et al., 1990; Kihara et al., 1992), PLA-N (Chijiwa et al., 2003), and PLA-B (Yamaguchi et al., 2001) are [Asp^{49}]PLA_2 forms, and the other three isozymes, BPI (Yoshizumi et al., 1990), BPII (Liu et al., 1990), and BPIII (Murakami et al., 2009) are [Lys^{49}] PLA_2 forms. They exert a variety of toxic effects their own such as myotoxicity (Kihara et al., 1992), neurotoxicity (Chijiwa et al., 2003), edema-inducing (Yamaguchi et al., 2001), *etc*. However, it is unclear how such venomous PLA_2s are specifically expressed only in the venom gland.

The venom gland-specific transcription factors are expected to play a key role for regulatory expression of the venom protein genes. In an effort to identify the venom gland-specific transcription factors, a full-length cDNA library for *P. flavoviridis* venom gland was constructed after milking the venom since it is thought that expression levels of transcription factors must be elevated at the stage replenishing the venom. A bioinformatic approach was employed to find transcription factors from the full-length cDNA library. Then, we discovered several full-length cDNAs encoding proteins with homology to epithelium-specific ETS (E twenty-six) transcription factors.

The ETS family is one of the largest transcription factor families, and all ETS family proteins share a highly conserved DNA binding domain (ETS domain), which forms a winged helix-turn-helix structure that binds to core 5′-GGAA/T-3′ sequences (Karim et al., 1990; Nye et al., 1992). Within the ETS family, the members that are specifically expressed in epithelial-rich tissues are classified into epithelium-specific ETS (ESE) subfamily. ESE-1, ESE-2, and ESE-3 are the members of the subfamily, all of which can be clustered together as a distinct subgroup based on phylogenetic consideration of the sequences of their ETS domains (Kas et al., 2000). ESE-1 is broadly expressed in tissues, such as lung, liver, kidney, colon, small intestine, pancreas, and prostate (Oettgen et al., 1997). ESE-2 and ESE-3 are expressed in the tissues that are particularly enriched in glandular epithelia, such as salivary gland, mammary gland, and prostate (Oettgen et al., 1999; Kas et al., 2000).

In the work reported here, we examined whether ESE subfamily proteins expressed in *P. flavoviridis* venom gland are capable of activating the promoters of venom PLA_2 genes, which are regarded as the representatives of the genes expressed in the venom gland. We also examined the binding specificities of ESE-3 to the ETS binding motifs located near TATA box in the promoters of venom PLA_2 genes.

2. Materials and methods

2.1. Materials

Various tissues including a venom gland were excised from *P. flavoviridis* snakes captured in Amami-Oshima island, one of the southwestern islands of Japan. Chinese hamster ovary CHO-K1 cells were supplied from RIKEN BioResource Center (Ibaraki, Japan). Unlabeled oligonucleotides and fluorescein isothiocyanate (FITC) end-labeled oligonucleotides were purchased from Genenet (Fukuoka, Japan) and Tsukuba oligo service (Ibaraki, Japan), respectively.

2.2. Preparation of full-length cDNA library of P. flavoviridis venom gland and the sequencing

A *P. flavoviridis* venom gland was excised about 20 h after its venom had been milked and employed for constructing its full-length cDNA library. The total RNA was extracted from the venom gland with RNeasy kit (Qiagen, Hilden, Germany). The full-length cDNA library was constructed from the total RNA ligated into pGCAP10 vector by Hitachi Hitech Ltd, the contracted research company, in Japan.

The colonies from the full-length cDNA library were randomly picked and inoculated into 384 microwell plates using a colony picker (Flexys; Genomic Solution). The sequencing templates were prepared from these arrayed cultures by using a TempliPhi DNA Amplification Kit (GE Healthcare, Buckinghamshire, UK). The EST (expressed sequence tag) sequencing reaction was carried out by using a BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster city, CA, USA). The sequence primer employed for the 5′ sequencing was 5′-AGGCCTGTACG-GAAGTGT-3′ located in the 5′ flanking region close to multicloning site in pGCAP10, and the primer for the 3′ sequencing was 5′-TTTTTTTTTTTTTTTTTTTTTTV-3′. The products of the sequencing reaction were purified by ethanol precipitation, and then loaded onto ABI 3730 DNA sequencers. Raw sequence data were base-called by the KB basecaller program. For detection of the vector sequence, the cross-match program was used. The nucleotide sequences of full-length cDNAs encoding ESE subfamily proteins and β-actin were determined by the primer walking method. Their nucleotide sequences are available from the GenBank/EMBL/DDBJ databases under accession nos. AB917067 (ESE-1), AB917068 (ESE-2), AB917069 (ESE-3), and AB972439 (β-actin) (Fig. S1).