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Accelerated evolution of small serum proteins (SSPs)—The PSP94 family proteins in a Japanese viper☆

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ABSTRACT

Five small serum proteins (SSPs) with molecular masses of 6.5–10 kDa were detected in Habu (*Trimeresurus flavoviridis*) serum; this included two novel proteins SSP-4 and SSP-5. The amino acid sequences of these proteins and of SSP-1, SSP-2, and SSP-3, which were reported previously, were determined on the basis of the nucleotide sequences of their cDNAs. Although these proteins exhibited only limited sequence identity to mammalian prostatic secretory protein of 94 amino acids (PSP94), the topological pattern of disulfide bonds in SSPs was identical to that of the mammalian proteins. SSP-3 and SSP-4 lacked approximately 30 residues at the C-terminal. Each of the full-length cDNAs encoded a mature protein of 62–90 residues and a highly conserved signal peptide. The evolutionary distances between SSPs estimated on the basis of the amino acid changes were significantly greater than those of the synonymous nucleotide substitutions; these finding, together with results from analyses of nonsynonymous to synonymous rates of change (dN/dS) suggest that snake SSPs have endured substantial accelerated adaptive protein evolution. Such accelerated positive selection in SSPs parallels other findings of similar molecular evolution in snake venom proteins and suggests that diversifying selection on both systems may be linked, and that snake SSP genes may have evolved by gene duplication and rapid diversification to facilitate the acquisition of various functions to block venom activity within venomous snakes.

1. Introduction

Proteins that inhibit snake venom hemorrhagic metalloproteinases (MPs) have been isolated from the sera of certain snakes; examples include HSF from the Habu, *Trimeresurus flavoviridis* (Yamakawa and Omori-Satoh, 1992), and BJ46a from *Bothrops jararaca* (Valente et al., 2001). Previously, we isolated a novel 10-kDa protein, SSP-1, having MP inhibitory activity from Habu serum (Aoki et al., 2007). Unlike other antihemorrhagic proteins, however, this so-called small serum protein (SSP) could only inhibit brevilysin H6, a weakly hemorrhagic MP isolated from *Gloydius halys brevicaudus* venom (Fujimura et al., 2000). Analysis of the amino acid sequence indicated that SSP belongs to the prostatic secretory protein of 94 amino acids (PSP94) protein family, based on topological similarities of the cysteine residues, although the sequence homology was low. Two additional similar proteins, SSP-2 and SSP-3, in the same serum from the *T. flavoviridis* were also isolated; SSP-2 has strong binding affinity to triflin, a smooth muscle contraction blocker isolated from *T. flavoviridis* venom (Yamazaki et al., 2002). This suggests that several distinct SSPs exist in snake blood where they are likely used as a defense against the toxic effects of their own venom which may circulate through the blood as the result of accidental self-envenomation (Aoki et al., 2007).

PSP94, also called β-microseminoprotein, is a 10.7-kDa, nonglycosylated, cysteine-rich protein (Dube et al., 1987). Even though PSP94 was first isolated as a major protein from human seminal plasma (Lilja and Abrahamsson 1988), it was later found to be present at the same level in both sexes (von der Kammer et al., 1990). PSP94 proteins have also been identified in other mammals (Fernlund et al., 1994; Xuan et al., 1999; Mäkinen et al., 1999) and in ostrich (Lazure et al., 2001). However, the amino acid sequences of these PSP94 family proteins show a limited homology. Apart from the 10 cysteines that form five disulfide bonds, there are only 16 amino acids conserved in the mammalian PSP94 family proteins. This suggests that these proteins are quite divergent and may be functionally quite different than the human PSP94 (Mäkinen et al., 1999; Nolet et al., 1991).

Genes for some snake venom proteins from *T. flavoviridis* (Ogawa et al., 1995; Nakashima et al., 1995; Chijiwa et al., 2003), *Naja naja* (Chuman et al., 2000), and *Ophiophagus hannah* (Chang et al., 2001) have been reported to evolve rapidly by gene duplication followed by

Abbreviations: cDNA, DNA complementary to RNA; CRISP, cysteine-rich secretory protein; HPLC, high performance liquid chromatography; MP, metalloproteinase; ORF, open reading frame; PLA_2, phospholipase A_2; PSP94, prostatic secretory protein of 94 amino acids; PAGE, polyacrylamide gel electrophoresis; PCR, polymerase chain reaction; RACE, rapid amplification of cDNA ends; SDS, sodium dodecyl sulfate; SSP, small serum protein; UTR, untranslated region.

☆ The nucleotide sequence data reported here have been deposited to the DDBJ sequence data bank: SSP-1, AB360906; SSP-2, AB360907; SSP-3, AB360908; SSP-4, AB360909; SSP-5, AB360910.

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rapid accumulation of nonsynonymous nucleotide substitutions that result in amino acid replacements in the encoded proteins. This type of molecular evolution results in the accelerated creation of toxins with diverse functions (Ohno et al., 1998).

In this study, we isolated two additional SSPs, SSP-4 and SSP-5, from the serum of *T. flavoviridis*. We also isolated a series of cDNA clones encoding the five SSPs from the liver of the same species, and determined the nucleotide sequences of these cDNAs. Based on these cDNA sequences, we investigated molecular evolutionary patterns in these SSP genes. Our molecular evolutionary analyses suggest that unusually rapid evolution of the amino acid sequences of snake SSP proteins has occurred, possibly driven by selection for functional diversification. Such radical rapid evolution of SSP proteins parallels numerous reports of similarly accelerated evolution of snake venom proteins, and suggests a potential coevolutionary interaction between these two functionally related classes of snake proteins.

2. Materials and methods

2.1. Materials

The blood and liver of *T. flavoviridis* were obtained from specimens collected from the Amami Oshima Islands. The sera obtained from five animals were pooled and stored at −20 °C. RNA was extracted from a single snake. Enzymes used for RNA and DNA manipulation were obtained from Takara Shuzo (Kyoto) except for PowerScript reverse transcriptase (Clontech). All other reagents were purchased from Wako Pure Chemicals (Osaka).

2.2. Purification of SSPs

The *T. flavoviridis* serum (30 ml) was loaded onto a Sephacryl S-200HR column (5×90 cm). Elution was carried out at 4 °C with 0.15 M NaCl–50 mM Tris–HCl (pH 7.4). Fractions containing SSPs were collected, desalted by dialysis, and subjected to reverse-phase HPLC on a Cosmosil-5C8-AR-300 column (1×25 cm, Nacalai Tesque). Elution was carried out with an appropriate gradient of acetonitrile in 0.1% trifluoroacetic acid (TFA) at a flow rate of 3.0 ml/min, and the eluate was monitored at 230 nm.

2.3. Electrophoresis

SDS-PAGE was carried out on a 16.5% polyacrylamide gel under nonreducing conditions (Schagger and Jagow, 1987). Ovalbumin (46,000), carbonic anhydrase (30,000), chymotrypsinogen (25,000), soybean trypsin inhibitor (20,500), lysozyme (14,300), aprotinin (6500), and insulin B-chain (3500) were used as the molecular weight markers. After running the gels under constant current, they were stained with 0.1% Coomassie brilliant blue R-250 and destained with 10% acetic acid.

2.4. Mass spectrometric analysis

The mass spectrum was measured on a Voyager DE-STR matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometer (PerSeptive Biosystems). The sample was dissolved in 0.1% TFA-50% acetonitrile containing α-cyano-4-hydroxycinnamic acid (10 mg/ml) as the matrix. The spectrum was calibrated on the basis of the molecular mass of apomyoglobin.

2.5. Sequence analysis

The protein was *S*-pyridylethylated (Friedman et al., 1970), and the amino acid sequence of the modified protein was determined by an automatic protein sequencer PPSQ 21 (Shimadzu).

2.6. Cloning of cDNAs encoding SSP-4 and SSP-5

Total RNA was extracted from 0.5 g of *T. flavoviridis* liver using the acid guanidinium-phenol-chloroform method, and this RNA was then reverse transcribed to synthesize the first cDNA strand using an adaptor-linked oligo(dT) primer (5′-GGCCACGCGTCGACTAGTAC-$(dT)_{17}$-3′). The obtained cDNAs were used as templates for the 3′-RACE reaction. The cDNA cloning experiments for SSP-1, SSP-2, and SSP-3 have been described previously (Aoki et al., 2007). Synthetic oligonucleotides, i.e., *ssp-4N* (5′-TNCCNGARAAYGARGAYGGN-GARGAYGTNCCN-3′), *ssp-5N* (5′-NTGYTTYCARGGNWSNTTY-GARGCNAARMGNATG-3′), and 3′-*adp* (5′-GGCCACGC-GTCGACTAGTAC-3′), were used for PCR amplification. The *ssp-4N* and *ssp-5N* primers were designed on the basis of the N-terminal amino acid sequences of SSP-4 and SSP-5, and 3′-*adp* corresponded to the adaptor sequence within an adaptor-linked oligo(dT) primer. The amplification products were subcloned into the plasmid vector, and their nucleotide sequences were determined. The results confirmed that they were partial fragments of cDNAs encoding SSP-4 and SSP-5. These fragments were radiolabeled with [^{32}P]-dCTP (3000 Ci/mmol) using a random primer DNA labeling kit (Takara Bio) and then used for hybridization screening of a cDNA library.

The *T. flavoviridis* liver cDNA library was constructed using the Creator SMART cDNA Library Construction kit (BD Biosciences) according to the manufacturer's instructions. Briefly, the first cDNA strand was synthesized using 1 μg of total RNA, and this was followed by five PCR cycles for nonspecific enrichment of full-length cDNAs. The cDNA fragments were then ligated to the pDNR-LIB vector. When the plasmid clones were used to transform *Escherichia coli* JM109, the resulting library contained 2.0×10^6 independent clones.

Clones (5×10^4) from an unamplified cDNA library were plated on LB agar. The bacterial colonies were transferred onto Hybond-NX membranes (GE Healthcare Bio-Science) and fixed by UV irradiation. The resulting replica membranes were prehybridized in Church's hybridization solution at 65 °C for 30 min and then hybridized overnight at 50 °C with either radiolabeled *ssp-4* or *ssp-5* cDNA in Church's hybridization solution, respectively. The membranes were washed twice for 15 min at 50 °C with 1× SSC containing 0.1% SDS, and the hybridization signals were visualized using a BioImage Analyzer (Fuji Film). Finally, 30 bacterial colonies were isolated and cultured. Their plasmids were purified by the standard alkali-SDS method. The nucleotide sequences of the cDNA inserts were determined using the ABI PRISM 377 DNA Sequencing System (Applied Biosystems).

2.7. Comparison and phylogenetic analysis

The nucleotide sequences were aligned and compared using DNASIS (Hitachi software engineering). Phylogenetic analysis was carried out by using CLUSTALW on the DNA Database Japan (DDBJ) web page and MEGA4 (Tamura et al., 2007); phylogenetic trees were drawn using TreeView (http://taxonomy.zoology.gla.ac.uk/rod/rod.html). Detection of positive selection along phylogenetic lineages was performed using GA-Branch, a codon-based genetic algorithm available on the Datamonkey web server (http://www.datamonkey.org; Kosakovsky Pond and Frost, 2005).

Previously reported cDNA and genomic sequences encoding PSP94 family proteins were obtained from GenBank and used in comparative analyses: human (accession No. S67815), rhesus monkey (M92161), cotton-top tamarin (AJ010154, AJ010158, and AJ010156 for *mspE1*, *mspA1*, and *mspJ1*, respectively), pig (NM_213852), rat (U65486), mouse (J89840), chicken (XM_421645), African clawed frog (AW641318), zebrafish (XM_001332586), Japanese flounder (C23089), cow (XM_867982), and horse (XM_001493992).