Review

Evolution of Phospholipase A_2 Toxins in Venomous Animals

Dušan Kordiš

Department of Molecular and Biomedical Sciences, Jožef Stefan Institute, Jamova 39, SI-1000 Ljubljana, Slovenia

** Corresponding author: E-mail: dusan.kordis@ijs.si*

Received: 06-06-2011

Dedicated to the memory of Professor Franc Gubenšek

Abstract

Franc Gubenšek devoted much of his research career to the phospholipases A_2 (PLA_2), which are the major pharmacologically active components of snake venoms. Our long collaboration started with an analysis of *Vipera ammodytes* ammodytoxin and ammodytin cDNAs and genes. These PLA_2 genes provided us with an entry into the exciting area of molecular evolution. We studied the structures of the PLA_2 genes, the evolution of multigene families encoding PLA_2 toxins, and the horizontal transfer of unusual retroelements that we found in these genes. In the last decade a number of novel features have emerged concerning the evolution of PLA_2s in venomous animals. The large amount of recently accumulated data has provided a timely opportunity to review current understanding of the evolution of PLA_2 toxins in venomous animals.

Keywords: Phospholipase A_2, venom, adaptive evolution, neofunctionalization

1. Introduction

Venom phospholipases A_2 (PLA_2) induce numerous pharmacological effects – neurotoxic, myotoxic, cardiotoxic, anticoagulant, antiplatelet, haemolytic, hemorrhagic, convulsive, hypotensive, inflammatory and local tissue damage.[1] They target a specific organ or tissue by high affinity binding to specific proteins. This specific binding is conferred by the presence of a "pharmacological site" on the protein's surface which is independent of the catalytic site.[2] The phenomenon of accelerated evolution in the PLA_2 toxins was first observed in the cDNAs encoding PLA_2 isozymes from *Protobothrops flavoviridis* venom gland.[3] Since then, accelerated evolution of venom PLA_2 toxins has been demonstrated in other Crotalinae, Viperinae, and Elapidae snakes.[4–15] The extraordinary level of positive selection acting on snake venom phospholipase A_2 genes indicates that adaptive molecular evolution plays an important role in the emergence, diversification and refinement of numerous pharmacological functions, and may contribute to niche differentiation following speciation.[16]

Franc Gubenšek devoted much of his research career to the PLA_2 toxins as one of the major pharmacologically active components of *V. ammodytes* venom.[17] Our long collaboration started with an analysis of the *V. ammodytes* cDNA library, in which the sequences of the cDNAs encoding the ammodytoxins A,[18] B[19] and C[20] were determined. Later we cloned ammodytoxin and ammodytin genes from the *V. ammodytes* genomic library.[21,22] These PLA_2 genes were quite interesting and provided us with an entry into the exciting area of molecular evolution.

With Franček we studied the structures of the PLA_2 genes, the evolution of multigene families encoding PLA_2 toxins, and horizontal transfer of the unusual retroelements that we found in these genes. Comparison of the Crotalinae and Viperinae genes and cDNA sequences has shown that 40 bp from the 3' end of exon 1 are deleted in all Crotalinae PLA_2 genes.[21] We demonstrated that the deleted exon segment is responsible for the intron retention within the 5'-UTR of their mRNAs and explained why Crotalinae group II PLA_2 genes[6] do not conform to the 5 exon/4 intron structural organization of all other group II PLA_2 genes.[21] The pattern of nucleotide substi-

tutions in protein-coding exons of *V. ammodytes* PLA_2 genes was found to be nonrandom and occurs preferentially on the first and the second positions of codons, suggesting positive Darwinian evolution for a new function.[23]

In the fourth intron of the AtxC and AtnL genes we found the highly conserved Ruminantia-specific Bov-B LINE retroposon.[22–24] The high level of sequence identity of the introns, the conservation of both flanking and untranslated regions, and the identical positions of truncated Bov-B LINES in both genes indicate that they have arisen by duplication and divergence of a common ancestral gene. The discovery of the Bov-B LINE in *V. ammodytes* and other snake genomes was of considerable interest, because it provided the first evidence for horizontal relationships of LINEs in vertebrates.[22,24–29]

The lengths of Viperidae PLA_2 genes, including proximal parts of promoter and 3' flanking regions, are between 2.0 and 2.7 kb.[21,22] Since the 5' and 3' flanking regions are highly conserved in all Viperidae group II PLA_2 multigene families, we designed oligonucleotide primers that enable amplification of the whole PLA_2 multigene family in a single step.[12] Using this approach we cloned two *Daboia palaestinae* PLA_2 genes, (VP7 (AF091855) and VP8 (AF091854)), two *Echis coloratus* PLA_2 genes – EC1 (AF253050), and EC3 (AF253049) – and also *V. ammodytes* ammodytin I1 (AF253048) and ammodytin I2 (X84018) genes that were found to be much older than myotoxic ammodytin L and neurotoxic ammodytoxin genes.[21,22]

Venom PLA_2 multigene families contain a variety of PLA_2 paralogs with diverse pharmacological activities acquired during the course of evolution.[23,30,31] The evolutionary history of snake venom PLA_2 multigene families can be regarded as the evolution from a single, general purpose (digestive), ancestral PLA_2 gene to multiple, increasingly diversified PLA_2s (e.g. myotoxins, neurotoxins) through a process of repeated gene duplication.[21–23,30] The number of PLA_2 genes in a single snake species indicates that several gene duplications have occurred during the evolution of PLA_2s in most snake species following speciation.[21–23,30,31]

These early evolutionary studies of snake group II PLA_2s also promoted the gene and evolutionary analyses of other venom components[30–34] and diverse large scale genome analyses concerning transposable elements[29,35–40] or large protein superfamilies.[41–43]

In our previous reviews of the evolution of snake PLA_2 toxins[23] and of the functional diversification of animal toxins by adaptive evolution[30,31] we provided an in-depth insight into these topics. In the last decade a number of studies have been reported concerning the evolution of PLA_2 toxins in venomous animals and the large amount of recently accumulated data has provided a timely opportunity to review current knowledge in this field.

2. Numerous Recruitment Events Leading to PLA_2 Toxins

Numerous proteins have been recruited convergently into the venoms of various animals. Molecular phylogenetic reconstruction of toxin evolutionary history has been used to illustrate the diversity within the integrated weapons system and to map the timing of toxin recruitment events over the organismal evolutionary tree.[44] Venom proteins are the result of toxin recruitment events in which an ordinary protein gene, typically one involved in a key regulatory process, is duplicated, and the new gene is selectively expressed in the venom gland.[44] After their recruitment, venom proteins evolve rapidly by a "birth and death" model of evolution, whereby frequent duplication of protein-encoding genes permits rapid functional and structural diversification alongside enhanced rates of sequence evolution.[30,32,46,47] Although some genes were deleted from the genome or degenerated into pseudogenes, others have undergone neofunctionalization, resulting in the generation of functionally diversified proteins.[16,30,31,45] Toxin multigene families preserve the molecular scaffold of the ancestral protein, but key functional residues outside the core scaffold are often modified to acquire numerous novel pharmacological activities.[30,31,45] Convergently recruited proteins share several conserved features: a secretory protein ancestor, functionally versatile protein ancestors with a fundamentally conserved basal activity, extensive disulfide cross-links, stable molecular scaffolds and, once recruited, adaptive evolution generates a suite of novel isoforms with neofunctionalization. The convergent origin of toxins across the entire metazoan spectrum suggests that there are functional and/or structural constraints on the evolution of animal venoms.[45]

PLA_2 enzymes have been recruited convergently into cnidarians, multiple insect orders, arachnids (scorpions, spiders, and ticks), cephalopods, and reptiles (twice into the advanced snake venoms and once into anguimorph lizard venom). Four PLA_2 scaffolds have been recruited into venoms – group I (G1), group II (G2), group III (G3) and group IX (G9).[16,45] Snake PLA_2 toxins originated by two independent recruitment events,[48] confirming existing information on the evolution of the toxins independently in elapid (PLA_2G1) and viperid venoms (PLA_2G2).[49,50] The recruitment of PLA_2G2 in viperids was closely linked to the evolution of advanced venom delivery systems and ambush feeding.[51] In contrast to the snakes, PLA_2G1s have undergone much more complex and dynamic evolution in invertebrates by numerous gene duplications, resulting in the greatest diversity of PLA_2G1s in invertebrate genomes.[33] Phylogenetic analysis has provided evidence that invertebrates possess numerous species-specific multigene families that evolved from a single ancestral PLA_2G1 and became highly diversified by adaptive evolution, like PLA_2s in snake venoms.[33] PLA_2G3s have been recruited independently into five venomous lineages, into cnida-

rians, insects, arachnids, cephalopods and anguimorph lizards, while PLA_2G9s have been recruited at least twice into cnidarians and into molluscs.[45]

3. Neofunctionalization and Adaptive Evolution in Snake PLA_2 Toxins: New Insights Obtained in the Last Decade

Neofunctionalization in PLA_2 toxins is defined as the emergence of a new toxic effect from an ancestral enzyme that did not possess that effect as its main toxin function.[16] Although previous studies of PLA_2 genes identified positive Darwinian selection in viperid PLA_2G2[6,12] and elapid PLA_2G1[14] genes, these studies focused only on one or two species, included relatively few genes and used methods that lack power to detect episodic adaptive evolution.

Snake venom PLA_2s are members of large multigene families with diverse pharmacological activities including neurotoxic, myotoxic, cardiotoxic, anticoagulant and haemolytic effects.[1] These diverse activities evolved from an ancestral nontoxic PLA_2 by a process of repeated gene duplication followed by functional divergence. Diverse pharmacological activities of snake venom PLA_2s must have originated in PLA_2 genes after gene duplications that occurred after they diverged from nontoxic ancestors.[16] It has been found that increase in genomic complexity (through gene duplications) can lead to phenotypic complexity (venom composition) and that positive Darwinian selection is a common evolutionary force in snake venoms.[16]

Recently, it has been shown that positive Darwinian selection and neofunctionalization is common in snake venom PLA_2 genes and is associated with the evolution of new toxin functions and speciation events, demonstrating that molecular adaptation has played a pervasive role in the evolution of snakes and their venom arsenal.[16] The pattern of gene duplication and positive selection indicates that adaptive molecular evolution occurs immediately after duplication events as novel functions emerge, and continues as gene families diversify and are refined.[16]

Maximum likelihood models of coding-sequence evolution have been used to test the hypothesis that functional diversification of snake venom PLA_2 genes was driven by positive Darwinian selection.[16] The estimate of $\omega = 1.28$ for PLA_2G1 genes under the one-ratio model is an average over all codons and lineages, highlighting the dominant role of positive selection on elapid venom PLA_2s. The estimate of $\omega = 0.686$ for PLA_2G2 genes under the one-ratio model indicates that PLA_2G2 genes are generally under purifying selection, however, this estimate is higher than those reported from most genes.[16]

A free-ratio model that estimates separate dN/dS ratios for all lineages in a tree was used to test the episodes of positive selection in PLA_2G1 and PLA_2G2 gene lineages.[16] These models fit the data significantly better than the one-ratio and constrained one-ratio models with ω forced to be 1 (PLA_2G1 genes), or a free-ratio model with lineages previously identified with $\omega > 1$ constrained to be 1 (PLA_2G2 genes), indicating that episodes of directional selection are common in snake venom PLA_2 evolution with nearly 32% and 21% of PLA_2G1 and PLA_2G2 gene lineages being under directional selection.[16]

Several branches with extremely high ω values were found, including two PLA_2G1 and three PLA_2G2 branches with $\omega > 3$, one PLA_2G1 branch with $\omega > 5$ and one PLA_2G1 branch with $\omega = 9.06$.[16] Nearly identical ω values of post-duplication (PD) and post-speciation (PS) branches have been found in PLA_2G1 genes, indicating that positive selection is associated with both gene duplication and speciation. In contrast to PLA_2G1 genes, PLA_2G2 gene PD branches evolve much faster than PS branches, consistent with the classical model of neofunctionalization.[16] Strikingly, positive selection occurred in the stem-lineage of 67% of PLA_2G1 functional groups and 88% of PLA_2G2 functional groups, indicating that positive selection played a pervasive role in the origin of novel toxin functions during the diversification of vipers and elapids and their venoms.[16]

Up to 65% of sites in PLA_2G1genes and 27% of sites in PLA_2G2 genes have been identified to be under positive selection.[16] This is strong evidence that diversification of snake venom PLA_2 genes is driven by recurrent positive selection and suggest that venomous snakes are caught in a co-evolutionary arms race with prey – as prey evolves resistance to the current venom arsenal and snakes evolve ever more toxic venoms.[16] The molecular evolution of PLA_2G1 and PLA_2G2 genes is characterized by the "birth and death" and "selective sieve" processes of gene duplication, divergence and loss. Such a pattern suggests a scenario where dietary shifts following speciation run the PLA_2 gene repertoire through a "selective sieve" – those genes that are no longer effective in subduing new prey species are lost, while genes that are still effective adapt to the new prey type and subsequently diversify.[16]

4. Regions Under Positive Selection are Located on the Surface of PLA_2 Proteins and Produce a Wide Spectrum of Pharmacological Effects

PLA_2 toxicity is independent of enzymatic activity and is mediated through "pharmacological sites" on the protein surface that interact directly with ligands on the

cell membrane.[1,2] The surface of PLA_2s therefore forms a scaffold for adaptive modification that has been used to generate a diverse array of pharmacological effects through a process of neofunctionalization.[16] It has been found that amino acid substitutions in PLA_2G1 and PLA_2G2 genes correlate with surface accessibility,[47] suggesting that modifications of surface residues and positive selection play important roles in generating toxin diversity.

To investigate how functional diversity is generated in PLA_2 enzymes, sites that were identified as being under diversifying selection were mapped on to the crystal structure of PLA_2G1 and PLA_2G2s.[16] The vast majority of amino acids under diversifying selection were found to be located outside the α-helical central scaffold and in regions of the protein that form connecting loops. The scaffold was found to be more conserved in PLA_2G2 than in PLA_2G1 proteins. Functionally important residues, including cysteines that participate in disulfide bonds, the catalytic triad, the calcium-binding site and the hydrophobic channel, were found to be under strong structural and functional constraint because their dN/dS ratios are near zero. In contrast, several clusters of amino acids on the molecular surface are under intense diversifying selection in PLA_2G1 and PLA_2G2 proteins. These rapidly evolving regions were found to be similar to the pharmacological sites, suggesting that regions under positive selection on the protein surface are responsible for generating toxic functions.[16]

Although only a few changes on the surface are needed to evolve a new function, there are regions under strong structural/functional constraints that limit divergence, such as the hydrophobic core and patches of conservation on the surface. It was found that several basal clades in the PLA_2G2 genes with uncharacterized pharmacological effects exhibit strong evidence of selection and have many amino acid replacements that map to the surface, suggesting that they may have evolved novel functions.[16] It was proposed long ago that "target sites" on the surface of prey cells are recognized by "pharmacological sites" on PLA_2 enzymes.[2] These protein-protein interactions determine PLA_2 specificity by having complementary charges, hydrophobicities, and van der Waals contact surfaces. Entirely new functions originate after duplication through substitutions in pharmacological sites that alter binding specificities. Although most substitutions will probably disrupt binding specificity for the current target site, a few may create new interaction sites leading to the emergence of novel functions.[16]

A direct link between the evolutionary and the functional diversification of venom proteins in closely related Sistrurus rattlesnakes has been provided: positive selection is acting to adjust which prey tissues and/or species are targeted and how the proteins act on those tissues, and this variation may be largely related to anticoagulant and/or haemolytic activity. This study provided evidence that such selection also operates over much shorter evolutionary timescales involved in adaptive radiations consisting of small numbers of closely related species.[52]

5. High Rates of Gene Turnover in Snake PLA_2 Multigene Families

Venom toxins form multigene families that are unique bioweapons in the predator-prey arms race.[53] The "birth-and-death" model best describes the evolution of the large multigene families.[46] Such a mode of evolution generates a suite of toxins in order to allow predatory animals to adapt to a variety of different prey species.[54]

Key features of the rapid evolution of snake PLA_2 genes, such as the targets of selection, rates of gene turnover and functional diversity of toxins generated, have remained unclear, especially in the closely related species with divergent diets. The evolution of PLA_2 genes was therefore studied in the four closely related Sistrurus rattlesnake species that feed on different prey.[52] It was found that each taxon possesses four to seven distinct PLA_2 sequences, and phylogenetic analysis suggested that these sequences represent a rapidly evolving gene family consisting of both paralogous and homologous loci with high rates of gene gain and loss.[52] Both gene gain and loss and protein sequence evolution by positive selection were found to be important evolutionary forces driving adaptive divergence in venom proteins in closely related species of venomous snakes.[52] In closely related Sistrurus species, a "birth-death" mechanism has been shown to drive PLA_2G2 gene family evolution over very short evolutionary time scales, providing the first estimates of the rates of gene gain (0.40 locus/species/myr) and loss (0.37 locus/species/myr) for a venom PLA_2G2 gene family.[52] Direct evidence for an "extinction" event has been found in the form of a distinct PLA_2 DNA sequence that was identified by cloning from *S. c. edwardsii* and classified as a pseudogene due to the presence of a 37-bp frameshift insertion in an exon. Both rates are one to two orders of magnitude higher than mean estimates of rates of gene gain and loss based on studies of paralogous loci in humans.[52]

Evolutionary turnover in PLA_2 gene families through a birth-and-death process was found to be at least one order of magnitude greater than that found in most other gene families.[52] This implies that gene gain and loss constitute an important evolutionary force in the adaptive evolution of venom genes, even among closely related species of venomous snakes. The extremely high level of PLA_2 gene turnover hints that an unknown genetic mechanism may allow unusually high rates of venom gene turnover to occur, leading to exceptionally rapid adaptive divergence through gene gain and loss when speciation occurs.[52]

6. Molecular Mechanisms that Cause the Accelerated Evolution of PLA_2 Toxins and Mechanisms that Generate their Molecular Diversity

The molecular mechanisms that cause accelerated evolution – a bias towards nucleotide mutations that lead to amino acid changes (non-synonymous substitutions), as compared to those that do not (synonymous substitutions) – are currently not understood. Interestingly, nucleotide sequences appear to determine the accelerated rate of point mutations. The distribution of stable and unstable nucleotide triplets was analyzed in PLA_2 genes.[55] It was found that toxin genes contain a higher percentage of unstable triplets in their exons than in introns, whereas non-toxin genes contain a higher percentage of unstable triplets in their introns. When trinucleotide sequences were considered, ten triplets showed mutation rates that were higher (>40%) than those of other triplets. Not surprisingly, all eight combinations containing CG dinucleotides showed higher mutation rates. It was suggested that specific nucleotide sequences affect mutation rates in PLA_2 genes.[55] The stable triplets were found more commonly in introns, and the unstable triplets more commonly in exons. The distribution of individual triplets also follows a similar trend; five out of eight stable triplets were more commonly found in introns than exons, whereas five out of eight unstable triplets were predominant in the exons. Thus the distribution of stable and unstable triplets is correlated with, and may explain, the accelerated evolution of exons in PLA_2 genes. The proteins expressed in the venom gland, compared to those expressed elsewhere in the snakes, have higher levels of unstable and less stable triplets in exons. It was suggested that specific nucleotide sequences in the toxin genes may determine the rate of evolution in the corresponding proteins.[55]

Molecular diversity of venom toxins can be generated by several mechanisms.[16,23,30,31,45] A recently discovered mechanism, termed accelerated segment switch in exons to alter targeting (ASSET), may play an important role in generating the molecular diversity in snake venom molecules.[56] During ASSET, certain parts of exons are changed through accelerated segment switch and generate a functionally new toxin with a conserved structural fold. Recently, it was shown that, in viperid venom genes, exons undergo rapid changes through accelerated segment switching, in contrast to their introns. Such an accelerated segment switch in exons can lead to change in the surface properties and hence functional diversification.[56] ASSET can lead to replacement of some of the critical amino acid residues that affect the biological function in venom toxins as well as changing the conformation of the loops that are involved in binding to specific target sites. Changes near the substrate binding regions are known to affect substrate specificity, and such exchanges may have significant implications for differences in isoform catalytic activity on specific target protein substrates. ASSET therefore plays an important role in functional diversification of snake venom proteins, in addition to accelerated point mutations in the protein coding regions. Accelerated point mutations lead to fine-tuning of target specificity, whereas ASSET leads to large-scale replacement of multiple, functionally important, residues, resulting in change or gain of functions.[56] Comparison of the amino acid sequences of PLA_2 toxins has revealed that the N-terminal region is undergoing exchange of a 13–14 amino acid long segment (forms helix B in several PLA_2 toxins) that lies between the first helix and the calcium-binding loop.[56] Differences in exchange within the same species appear to arise due to ASSET, as there is more than one amino acid replacement. The residues in this short segment have been proposed to play a crucial role in some of the pharmacological effects of PLA_2 enzymes. Accelerated point mutations result in fine modifications to the surface topology and/or electrostatic potential, whereas ASSET drastically alters the surface, virtually instantaneously producing large-scale changes in the ligand interaction site(s).[56] In PLA_2 toxins only one surface segment is changed by ASSET, but it most likely affects their functional properties. It was proposed that ASSET occurs first, resulting in drastic changes in functionally important surface regions, followed by accelerated point mutations in those regions that fine-tune the target specificity. Although the molecular mechanisms of ASSET and accelerated point mutations are unknown, both contribute to the evolution of snake venom toxins and both help to explain the observed functional diversity of toxins and the evolution of new functions in snake venom protein superfamilies.[56]

Alternative splicing (AS) plays a crucial role in the diversification of gene function and regulation. AS is one of the key mechanisms that have evolved in metazoans to generate increased transcriptome complexity, and recent studies estimate that more than 95% of human multi-exon genes express multiple splice isoforms.[57] Moreover, alternatively spliced exons are often differentially regulated across tissues and during development, suggesting that individual isoforms may serve specific spatial or temporal roles.[57] The rarest AS event in vertebrates and invertebrates is intron retention, in which an intron remains in the mature mRNA transcript, accounting for less than 5% of known AS events. By contrast, intron retention is the most prevalent type of AS in plants, fungi and protozoa.[57] The preponderance of retained introns in UTRs may indicate their regulatory role, for example, affecting RNA stability or translational efficiency.[58] Comparison of the Crotalinae and Viperinae PLA_2 genes has shown that Crotalinae PLA_2s retain the first intron in their mRNAs.[21] All splicing signals are conserved in the nearly 90% identical retained intron of Crotalinae PLA_2 genes. A deletion of 40 bp from the 3' part of exon 1 in all Crotalinae PLA_2 genes was evident. The deleted segment appears to be responsib-

le for the retention of the subsequent intron within the 5'-UTR of their mRNAs that are consequently longer by about 120 bp.[21] Analysis of the secondary structure of the pre-mRNA of the ammodytoxin C gene has shown that the first exon is able to form an intra-exon hairpin which is absent in Crotalinae PLA_2 pre-mRNAs. We demonstrated that 5' terminal exons and their secondary structures are involved in the aberrant pre-mRNA splicing, resulting in retention of the downstream intron.[21] Two alternative models of the 5' UTR intron effect on gene expression have been suggested.[59] The first is that splicing-dependent enhancement in gene expression is influenced not only by the position of an intron, but also by its size. The second model is that transcriptional regulatory proteins are recruited as a result of the presence of DNA elements, which in turn enhance expression level. This process could be restricted spatially, in such a way that, if the distance between the regulatory element and the transcription start site is long, the enhancement should be less pronounced. Hence, the genes with the highest expression levels might be under selective pressure to keep their introns short in order to retain their enhancer elements closer to the transcription start site. In this scenario, one can further imagine these elements to function in a tissue-specific regulatory mechanism if the recruited factors are themselves tissue-specific.[59] For 5' UTR introns, short intron length is an important predictor of high expression level.[59] The full genome-wide functional implications and importance of 5' UTR retained introns in Crotalinae PLA_2s need to be further addressed.

7. First Insight Into the PLA_2 Gene Locus of Venomous Snakes

In an attempt to elucidate the multiplication mechanism of PLA_2 genes, a 25 kb genome segment was obtained from Amami-Oshima *Protobothrops flavoviridis* and found to contain five PLA_2 genes (3 normal and 2 pseudogenes).[60] The PLA_2 genes in this cluster are PfPLA 1(ψ), PfPLA 2 encoding strongly myolytic [Lys49]PLA_2 and called BPII, PfPLA 3(ψ), PfPLA 4 encoding neurotoxic [Asp49]PLA_2, called PLA-N, and PfPLA 5 encoding edema-inducing basic [Asp49]PLA_2, called PLA-B. PfPLA 1(ψ) and PfPLA 3(ψ) are pseudogenes. Truncated CR1 LINEs were found close to the 3' end of the PLA_2 genes and were named PLA_2 gene-coupled RT fragments (Pc-RTFs). A recombination hotspot (37 bp long), named Scomb, was found 548 bp upstream of the TATA box of PLA_2 genes. It was concluded that multiple gene duplications of PfPLA-PfCR1 occurred by unequal crossing over of the segment "Scomb-PfPLA-PfCR1-Scomb". It was proposed that the *P. flavoviridis* PLA_2 gene cluster originated 40–50 million years ago, after the split of Crotalinae and Viperinae snakes. The tandemly organized PLA_2 gene cluster in *P. flavoviridis* genome was formed by consecutive gene duplication of an ancestral PfPLA-PfCR1 unit, as a result of unequal crossing over. FISH analysis of the chromosomes in metaphase with the gene probes, pgPLA 1a, pgPLA 1b, and BP-II labeled with biotin-16-dUTP, showed that all PLA_2 isozyme genes are located on a pair of microchromosomes in the *P. flavoviridis* genome.[60]

8. Influence of Diet on the PLA_2 Toxin Profile of Venomous Animals

The vast majority of venomous animals possess functionally diversified PLA_2 multigene families in response to the diet preference.[30] A few interesting exceptions have been found in the last decade and are described in more detail below.

Variation in PLA_2 venom proteins at inter- and intraspecific levels was found to be the result of diversification in PLA_2 genes by positive selection.[5,6,12,23] The composition of snake venom proteins is under strong natural selection for adaptation towards specific diets.[52,53,61,62] Prey capture is considered to be a major biological imperative driving the venom toxin selection process. The adaptations to diet occur within venom toxin families rather than resulting from changes in expression levels of entire toxin families.[63]

The link between PLA_2 gene and protein variation and different diets has been found among island populations of the habu snake.[64] The protein composition of venoms from *P. flavoviridis* of Okinawa island was different from that of snakes from the main island. Some of the main components, such as myotoxic PLA_2 enzymes BPI and BPII, were absent in Okinawa snakes. The genes encoding these proteins lost segments in their exon and intron and became pseudogenes. This loss of BPI and BPII genes may, however, not explain the overall decrease in the venom toxicity.[64]

Aipysurus eydouxii has adapted to a new dietary habit – feeding exclusively on fish eggs, the snake no longer uses its venom for prey capture.[54] This was paralleled by greatly atrophied venom glands and loss of effective fangs. It is interesting to note that a potent venom was not maintained for use in defense, thus reinforcing the assumption that the primary use of snake venoms is for prey capture. PLA_2 toxins from the *A. eydouxii* venom gland exhibit properties of accelerated evolution similar to those of other snake venom PLA_2 toxins. More substitutions occur in the protein-coding regions than in the UTRs, nonsynonymous substitutions occur more frequently than synonymous ones, and mutations occur more commonly on the molecular surface of the PLA_2 toxins.[54] Phylogenetic analysis has revealed a much lower rate of diversification of PLA_2 toxins in the *A. eydouxii* venom gland than in terrestrial and other marine snakes. In contrast to all the other snake venoms analyzed to date, gene duplication-diversification and accelerated evolution play a minimal ro-

le in the evolution of *A. eydouxii* PLA_2 toxins.[54] While the proteins are evolving via the birth-and-death model, births are few and the new toxins do not expand much and have premature deaths. It has been concluded that this slower evolution of PLA_2 toxins in *A. eydouxii* is the consequence of the recent dietary switch of the snake to a food source that does not require the use of venom for prey capture, and thus the positive pressure of natural selection on the mutation of *A. eydouxii* PLA_2 toxins was reduced significantly.[54] The loss of the main neurotoxin in *A. eydouxii* was accompanied by a much slower rate of molecular evolution of the PLA_2 toxins as a consequence of the snake's shift in ecological niche, which presents a unique situation. When its diet changed to fish eggs exclusively, the evolutionary rate slowed down as the positive pressure of natural selection dropped significantly. This kind of evolutionary mode (initial accelerated evolution, subsequent decelerated evolution) has been reported for the first time for any snake venom toxin.[54]

In *Austrelaps labialis* from Kangaroo Island an unusually accelerated rate of deletion and insertion was observed in toxin cDNA clones which has resulted in the death of functional toxin genes – among others, three PLA_2 genes were found to be truncated.[65] It has been proposed that a rapid rate of deletions/insertions, as with accelerated evolution of toxin genes, may have had a significant influence on the evolution and survival of *A. labialis*.[65] Some of the toxin transcripts have been found to be terminated prematurely, due either to insertion or to deletion of nucleotides. Due to these insertions and deletions some of the genes might not code for functional proteins. Such a higher rate of insertion and deletion might be responsible for its lower toxicity and play a crucial role in the evolution of toxin genes.[65]

PLA_2s are highly conserved in sea snakes, in contrast to land snakes or sea kraits, despite their extremely divergent and adaptive ecological radiation. Streamlining in habitat and diet in sea snakes has possibly kept their PLA_2 toxin genes conserved, suggesting that prey composition and diet breadth may contribute to the diversity and evolution of venom components.[66]

9. Regulatory Evolution of PLA_2 Toxins

The effects of gene regulation must now be considered as a potentially important explanation for the variation in abundance of different proteins in whole venom.[67] To date, much attention has been focused on how structural differences in proteins, due to positive diversifying natural selection in relation to diet, contribute to inter- and intraspecific variation in whole venom[30,52,53,61] and provide strong indirect evidence that such genetic effects are important. It was proposed that the abundant venom proteins may perform generic killing and digestive functions that are not prey specific, whereas less abundant proteins may be more plastic in either evolutionary or ecological timescales and serve to "customize" an individual snake's venom to feeding on a particular prey requiring a specific venom protein.[67] Given the insights arising from studies of the molecular evolution of venom gene coding regions, direct study of intra- and inter-specific polymorphism in the promoter regions upstream of coding regions of specific genes would be useful in exploring the possible genetic basis for regulatory effects.[67]

The promoter regions of some venom protein genes contain *cis*-regulatory elements that are reasonably well characterized.[68] A single transcription initiation site was found in *Naja sputatrix* PLA_2 genes.[69] Promoter sequences of the latter contain some consensus transcriptional factor binding-sites, such as TATA box, Sp1, γ -IRE and $(TG)_{12}$ repeat. Functional analysis of the promoter region of *N. sputatrix* PLA_2 genes in the CHO and HepG2 cell lines has shown that Sp1, AP2, γ -IRE and $(TG)_{12}$ repeats may be involved in the tissue-specific expression of these genes.[69] In all Viperidae PLA_2 genes, the proximal part of the promoter region is highly conserved, and all putative binding sites for transcription factors are in the same positions.[12] These putative binding sites and the promoter regions are almost 90% identical within all known PLA_2 genes of the Viperidae family. However, the sequence similarity is lost upstream from nucleotide –139. Comparative analyses on the Viperidae and Elapidae (*N. sputatrix*) PLA_2 genes show that there are some consensus regulatory elements in their promoter regions, but the sequence of the promoter region is less conserved.[69]

Insights into whether promoter regions of PLA_2 genes are undergoing selection could come from studies that determine levels of polymorphism in specific regulatory elements or from more general tests of whether the sequence in the promoter region is evolving under selection by comparing levels of polymorphism in the promoter and intronic regions.[67] Regulatory evolution should be considered as an important mechanism causing phenotypic variation in whole venom composition, and in which differential expression of different venom loci is important in producing species-specific venom that allows species to capture specific prey.[67]

10. Conclusions

The evolutionary analyses of the PLA_2 toxins in venomous animals took place in the pre-genomic era, from the early to late 90s, and were based on a small sample of taxonomic diversity and diversity within the PLA_2 toxins. Since then, the number of representatives has increased significantly, largely due to the accumulation of the venom transcriptomic resources since the larger genomic data regarding PLA_2 toxins in venomous animals are still very sparse. In this review, I have highlighted how the pro-

gress in the last decade has increased our understanding of the evolution of PLA_2 toxins in venomous animals. While much future work is still needed, the tools are all in hand, so we can expect increase in our understanding of the evolution of PLA_2 toxins in venomous animals in the foreseeable future.

11. Acknowledgements

I sincerely thank Prof. Roger H. Pain for critical reading of the manuscript. This study was supported by grant P1-0207 from the Slovenian Research Agency.

12. References

1. R. M. Kini, *Toxicon* **2003**, *42*, 827–840.
2. R. M. Kini, H. J.Evans, *Toxicon* **1989**, *27*, 613–635.
3. T. Ogawa, N. Oda, K. Nakashima, H. Sasaki, M. Hattori, Y. Sakaki, H. Kihara, M. Ohno, *Proc. Natl. Acad. Sci. U.S.A.* **1992**, *89*, 8557–8561.
4. H. Kihara, R. Uchikawa, S. Hattori, M. Ohno, *Biochem. Int.* **1992**, *28*, 895–903.
5. K. Nakashima, T. Ogawa, N. Oda, M. Hattori, Y. Sakaki, H. Kihara, M. Ohno, *Proc. Natl. Acad. Sci. U.S.A.* **1993**, *90*, 5964–5968.
6. K. Nakashima, I. Nobuhisa, M. Deshimaru, M. Nakai, T. Ogawa, Y. Shimohigashi, Y. Fukumaki, M. Hattori, Y. Sakaki, S. Hattori, M. Ohno, *Proc. Natl. Acad. Sci. U.S.A.* **1995**, *92*, 5605–5609.
7. T. R. John, L. A. Smith, I. I. Kaiser, *Gene* **1994**, *139*, 229–234.
8. T. Ogawa, K. Nakashima, N. Oda, Y. Shimohigashi, M. Ohno, S. Hattori, M. Hattori, Y. Sakaki, H. Kihara. *J. Mol. Recognit.* **1995**, *8*, 40–46.
9. T. Ogawa, K. Nakashima, I. Nobuhisa, M. Deshimaru, Y. Shimohigashi, Y. Fukumaki, Y. Sakaki, S. Hattori, M. Ohno, *Toxicon* **1996**, *34*, 1229–1236.
10. I. Nobuhisa, K. Nakashima, M. Deshimaru, T. Ogawa, Y. Shimohigashi, Y. Fukumaki, Y. Sakaki, S. Hattori, H. Kihara, M. Ohno, *Gene* **1996**, *172*, 267–272.
11. Y. Shimohigashi, A. Tani, Y. Yamaguchi, T. Ogawa, M. Ohno, *J. Mol. Recognit*. **1996**, *9*, 639–643.
12. D. Kordiš, A. Bdolah, F. Gubenšek, *Biochem. Biophys. Res. Commun.* **1998**, *251*, 613–619.
13. M. Ohno, R. Ménez, T. Ogawa, J. M. Danse, Y. Shimohigashi, C. Fromen, F. Ducancel, S. Zinn-Justin, M. H. Le Du, J. C. Boulain, T. Tamiya, A. Ménez, *Prog. Nucleic Acid. Res. Mol. Biol.* **1998**, *59*, 307–364.
14. Y. Chuman, I. Nobuhisa, T. Ogawa, M. Deshimaru, T. Chijiwa, N. H. Tan, Y. Fukumaki, Y. Shimohigashi, F. Ducancel, J. C. Boulain, A. Ménez, M. Ohno, *Toxicon* **2000**, *38*, 449–462.
15. B. S. Singh, A. Armugam, R. M. Kini, K. Jeyaseelan, *Arch. Biochem.* **2000**, *375*, 289–303.
16. V. J. Lynch, *BMC Evol. Biol.* **2007**, *7*, 2.
17. F. Gubenšek, A. Ritonja, J. Zupan, V. Turk, *Period. Biol.* **1980**, *82*, 443–447.
18. J. Pungerčar, D. Kordiš, B. Štrukelj, N. S. Liang, F. Gubenšek, *Toxicon* **1991**, *29*, 269–273.
19. D. Kordiš, J. Pungerčar, B. Štrukelj, N. S. Liang, F. Gubenšek, *Nucleic Acids Res.* **1990**, *18*, 4016.
20. J. Pungerčar, D. Kordiš, R. Jerala, M. Trstenjak-Prebanda, M. Dolinar, V. Čurin-Šerbec, R. Komel, F. Gubenšek, *Nucleic Acids Res.* **1989**, *17*, 4367.
21. D. Kordiš, F. Gubenšek, *Eur. J. Biochem.* **1996**, *240*, 83–90.
22. D. Kordiš, F. Gubenšek, *Eur. J. Biochem.* **1997**, *246*, 772–779.
23. F. Gubenšek, D. Kordiš, *Venom phospholipase A_2 genes and their molecular evolution,* in R. M. Kini (Ed.) Venom Phospholipase A_2 Enzymes: Structure, Function and Mechanism, Wiley, Chichester. **1997**, pp. 73–95.
24. D. Kordiš, F. Gubenšek, *Nat. Genet.* **1995**, *10*, 131–132.
25. D. Kordiš, F. Gubenšek, *Toxicon* **1998**, *36*, 1585–1590.
26. D. Kordiš, F. Gubenšek, *Proc. Natl. Acad. Sci. U.S.A.* **1998**, *95*, 10704–10709.
27. D. Kordiš, F. Gubenšek, *Gene* **1999**, *238*, 171–178.
28. D. Kordiš, F. Gubenšek, *Genetica* **1999**, *107*, 121–128.
29. V. Župunski, F. Gubenšek, D. Kordiš, *Mol. Biol. Evol.* **2001**, *18*, 1849–1863.
30. D. Kordiš, F. Gubenšek, *Gene* **2000**, *261*, 43–52.
31. D. Kordiš, I. Križaj, F. Gubenšek, *Functional diversification of animal toxins by adaptive evolution*. In: A. Menez (Ed.) Perspectives in Molecular Toxinology, Wiley, Chichester, **2002**, pp. 401–419.
32. V. Župunski, D. Kordiš, F. Gubenšek, *FEBS Lett.* **2003**, *547*, 131–136.
33. A. Razpotnik, I. Križaj, J. Šribar, D. Kordiš, P. Maček, R. Frangež, W. R. Kem, T. Turk, *FEBS J.* **2010**, *277*, 2641–2653.
34. S. Kauferstein, C. Porth, Y. Kendel, C. Wunder, A. Nicke, D. Kordiš, P. Favreau, D. Koua, R. Stöcklin, D. Mebs, *Toxicon* **2011**, *57*, 28–34.
35. N. Lovšin, F. Gubenšek, D. Kordiš, *Mol. Biol. Evol.* **2001**, *18*, 2213–2224.
36. B. Gorinšek, F. Gubenšek, D. Kordiš, *Mol. Biol. Evol.* **2004**, *21*, 781–798.
37. B. Gorinšek, F. Gubenšek, D. Kordiš, *Cytogenet. Genome Res.* **2005**, *110*, 543–552.
38. D. Kordiš, *Gene* **2005**, *347*, 161–173.
39. D. Kordiš, N. Lovšin, F. Gubenšek, *Syst. Biol.* **2006**, *55*, 886–901.
40. D. Kordiš, *Cytogenet. Genome. Res.* **2009**, *127*, 94–111.
41. T. Režen, N. Debeljak, D. Kordiš, D. Rozman, *J. Mol. Evol.* **2004**, *59*, 51–58.
42. M. Novinec, D. Kordiš, V. Turk, B. Lenarčič, *Mol. Biol. Evol.* **2006**, *23*, 744–755.
43. D. Kordiš, V. Turk, *BMC Evol. Biol.* **2009**, *9*, 266.
44. B. G. Fry, N. Vidal, L. van der Weerd, E. Kochva, C. Renjifo, *J. Proteomics* **2009**, *72*, 127–136.
45. K. Roelants, D. E. Champagne, H. Scheib, J. D. Tyndall, G. F. King, T. J. Nevalainen, J. A. Norman, R. J. Lewis, R. S.

Norton, C. Renjifo, R. C. de la Vega, *Annu. Rev. Genomics Hum. Genet.* **2009**, *10*, 483–511.
46. M. Nei, A. P. Rooney, *Annu. Rev. Genet.* **2005**, *39*, 121–152.
47. R. M. Kini, Y. M. Chan, *J. Mol. Evol.* **1999**, *48*, 125–132.
48. B. G. Fry, W. Wüster, *Mol. Biol. Evol.* **2004**, *21*, 870–883.
49. R. L. Heinrikson, E. T. Krueger, P. S. Keim, *J. Biol. Chem.* **1977**, *252*, 4913–4921.
50. M. J. Dufton, R. C. Hider, *Eur. J. Biochem.* **1983**, *137*, 545–551.
51. B. G. Fry, H. Scheib, L. van der Weerd, B. Young, J. McNaughtan, S. F. Ramjan, N. Vidal, R. E. Poelmann, J. A. Norman, *Mol. Cell Proteomics* **2008**, *7*, 215–246.
52. H. L. Gibbs, W. Rossiter, *J. Mol. Evol.* **2008**, *66*, 151–166.
53. J. C. Daltry, W. Wüster, R. S. Thorpe, *Nature* **1996**, *379*, 537–540.
54. M. Li, B. G. Fry, R. M. Kini, *Mol. Biol. Evol.* **2005**, *22*, 934–941.
55. R. M. Kini, A. Chinnasamy, *Toxicon* **2010**, *56*, 295–304.
56. R. Doley, S. P. Mackessy, R. M. Kini, *BMC Evol. Biol.* **2009**, *9*, 146.
57. H. Keren, G. Lev-Maor, G. Ast, *Nat. Rev. Genet.* **2010**, *11*, 345–355.
58. P. A. Galante, N. J. Sakabe, N. Kirschbaum-Slager, S. J. de Souza, *RNA* **2004**, *10*, 757–765.
59. C. Cenik, A. Derti, J. C. Mellor, G. F. Berriz, F. P. Roth, *Genome Biol.* **2010**, *11*, R29.
60. N. Ikeda, T. Chijiwa, K. Matsubara, N. Oda-Ueda, S. Hattori, Y. Matsuda, M. Ohno, *Gene* **2010**, *461*, 15–25.
61. A. Barlow, C. E. Pook, R. A. Harrison, W. Wüster, *Proc. Biol. Sci.* **2009**, *276*, 2443–2449.
62. H. L. Gibbs, S. P. Mackessy, *Toxicon* **2009**, *53*, 672–679.
63. N. R. Casewell, R. A. Harrison, W. Wüster, S. C. Wagstaff, *BMC Genomics* **2009**, *10*, 564.
64. T. Chijiwa, S. Hamai, S. Tsubouchi, T. Ogawa, M. Deshimaru, N. Oda-Ueda, S. Hattori, H. Kihara, S. Tsunasawa, M. Ohno, *J. Mol. Evol.* **2003**, *57*, 546–554.
65. R. Doley, N. N. Tram, M. A. Reza, R. M. Kini, *BMC Evol. Biol.* **2008**, *8*, 70.
66. S. Pahari, D. Bickford, B. G. Fry, R. M. Kini, *BMC Evol. Biol.* **2007**, *7*, 17.
67. H. L. Gibbs, L. Sanz, J. J. Calvete, *J. Mol. Evol.* **2009**, *68*, 113–125.
68. L. S. Chang, *Toxin Rev.* **2007**, *26*, 143–167.
69. K. Jeyaseelan, A. Armugam, M. Donghui, N. H. Tan, *Mol. Biol. Evol.* **2000**, *17*, 1010–1021.

Povzetek

Franc Gubenšek je posvetil večino svoje raziskovalne kariere fosfolipazam A_2 (PLA_2), ki so prevladujoča farmakološko aktivna sestavina kačjih strupov. Najino dolgo sodelovanje se je začelo z analizo cDNA in genov, ki kodirajo amoditoksine in amoditine pri modrasu (*Vipera ammodytes*). Ti PLA_2 geni so nam omogočili vstop na zanimivo področje molekularne evolucije. Proučevala sva strukturo PLA_2 genov, evolucijo multigenskih družin, ki kodirajo PLA_2 toksine ter horizontalni prenos nenavadnih retroelementov, ki sva jih našla v teh genih. V zadnjem desetletju so se pojavila številna nova odkritja, ki so povezana z evolucijo PLA_2. V tem preglednem članku je predstavljeno sedanje stanje poznavanja evolucije PLA_2 toksinov pri strupenih živalih.