Toxicity evolution of *Vipera aspis aspis* venom: identification and molecular modeling of a novel phospholipase A_2 heterodimer neurotoxin[1]

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Received 6 June 2002; revised 1 August 2002; accepted 2 August 2002

First published online 21 August 2002

Edited by Robert B. Russell

Abstract **We report the simultaneous presence of two phospholipase A_2 (PLA_2) neurotoxins in the venom of *Vipera aspis aspis*, the first such observation. One is monomeric and identical to ammodytoxin B of *Vipera ammodytes ammodytes*. Its presence may result from gene flux after interbreeding between *V. aspis aspis* and *V. ammodytes ammodytes*. The second, a novel heterodimer named vaspin, is very similar to vipoxin of *Vipera ammodytes meridionalis* and to PLA_2-I of *Vipera aspis zinnikeri*. It may result from expression of preexisting genes, the acidic subunit evolving from an ancestor common to ammodytin I2 from *V. ammodytes ammodytes*, which we also found in *V. aspis aspis*.**

Key words: Evolution; Interbreeding; Neurotoxin; Phospholipase A_2; Snake venom; Three-dimensional structure

1. Introduction

Three venomous snakes are of public health significance in Europe: *Vipera aspis* (Linnée, 1758), *Vipera berus* (Linnée, 1758) and *Vipera ammodytes* (Linnée, 1758). Envenomation by the most widespread vipers found in France, *V. aspis aspis* (Linnée, 1758) (*Vaa*) and *V. berus berus* (Linnée, 1758) (*Vbb*), generally causes local symptoms (pain, edema, phlyctenosis) associated in severe cases with systemic effects (gastrointestinal disorders and hypotension) [1]. In addition, neurological symptoms (diplopia, paralysis) have been observed following *V. ammodytes* or '*V. aspis zinnikeri* Kramer, 1958' bites (*Vaz*) [2].

The venoms of *Vaa* and *Vbb* are very similar in activity and contain proteins acting on hemostasis, inflammation and blood pressure [3–7]. The venom of *V. ammodytes* species differs from those of *Vaa* and *Vbb* in that it contains neurotoxic phospholipases A_2 (PLA_2) which may be either monomeric or heterodimeric [8,9]. Monomeric PLA_2s have been demonstrated in the venom of *V. ammodytes ammodytes* (Linnée, 1758) (*Vamam*): they consist of three isoforms, namely ammodytoxin A, B and C, and block acetylcholine release in neuromuscular junction. The heterodimeric PLA_2 vipoxin from *V. ammodytes* is a postsynaptic neurotoxin. It consists of a presynaptic neurotoxic basic protein with PLA_2 activity and a non-toxic, non-enzymatic, acidic protein that protects the basic subunit from degradation and modifies its pharmacological activity. Its subspecies origin is unclear. Recent papers suggested that it is a specific neurotoxin from *V. ammodytes meridionalis* Boulenger, 1903 [10,11] (*Vamme*) whereas previous studies described its isolation from the venom of *Vamam* snakes [12–14]. A similar heterodimeric neurotoxin has also been found in *Vaz* venom [15,16]. The neurotoxins found in the European viper subspecies and their sites of action are listed in Table 1.

Between 1992 and 2001, the Marseille Poison Center observed 11 cases of envenomation after *Vaa* bites in Southeast France (departments of Alpes-Maritimes and Alpes-de-Haute-Provence) involving unexpected neurological signs in addition to the more usual symptoms that were only mild in intensity [17,18]. The viper responsible for one of these 'neurotoxic' envenomations was captured alive and was classified as *Vaa* on the basis of its morphology. The composition pattern of its venom as assessed by polyacrylamide gel electrophoresis (PAGE) was similar to that of *Vaz* and different from that of the nominotypic *Vaa* [18]. This was surprising as recent herpetological data indicate that *Vaz* is only found in Southwest France [19]. Spread of the *Vaz* population is unlikely because there have been no neurotoxic envenomations between Montpellier (Hérault) and Nice (Alpes-Maritimes).

The numerous bites associated with neurological signs in this region suggest the appearance of a new population of snakes. Intraspecific variations in snake venom composition have often been described without associated differences in morphology between individuals [20]. In the *Vipera* genus, this phenomenon was first found in Southeast Europe (Bulgaria), then in Southwest (France) and recently in Asia (Taïwan) [21]. The causes of the changes in venom composition within a species or a subspecies are still unclear [22]. The neurotoxic components of the venom of vipers are all members of the group IIA PLA_2 family. PLA_2 genes undergo accelerated molecular evolution [23], so we investigated whether the variability of symptoms is associated with changes in venom composition.

Here, we report for the first time the presence of two neuro-

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0014-5793/02/$22.00
PII: S0014-5793(02)03205-2

toxins in the venom of *Vaa*: ammodytoxin B and a novel neurotoxin, vaspin, which is a non-covalent complex of one acidic and one basic group IIA PLA_2, analogous to vipoxin and PLA_2-I. Molecular modeling and structural analysis of vaspin suggest that this toxin is responsible for neurological symptoms, possibly by acting at postsynaptic neuromuscular junctions. The acidic subunit of vaspin appears to share a common ancestor with ammodytin I2 from *Vamam*.

2. Materials and methods

2.1. Materials

Total RNA was extracted in deionized water and 0.001% DEPC (ICN Biomedicals, USA), ethanol absolute from Merck (Darmstadt, Germany), propan-2-ol from Prolabo (Fontenay sous bois, France) and chloroform from Carlo Erba Reagenti (Val de Reuil, France). RT-PCR was performed with the Ready To Go™ You Prime First-Strand Beads product from Amersham Pharmacia Biotech (Piscataway, NJ, USA). PCR nucleotide mix from Promega (Madison, WA, USA) and Genset oligo primers (Paris, France) were used. Taq DNA polymerase and 10× buffer were purchased from Amersham Pharmacia Biotech. The equipment used included an Eppendorf mastercycler gradient machine for PCR, a Biolafitte table for shaking and an Eppendorf 5810R for centrifugation.

The study is based on the extraction of seven *Vaa* venom glands. Three of these snakes were captured in the department of the Puy-de-Dôme, where only classical cases of envenomations were reported, and used as controls. The four others were captured in the Alpes-Maritimes and the Alpes-de-Haute-Provence departments. One of the snakes was captured just after a bite in the Alpes-Maritimes and was responsible for neurological symptoms as described by de Haro et al. [17].

2.2. Methods

Snakes were identified by use of classical keys of determination (e.g., number of ventral and susocular plates, scale ranges between the labial plates and the eye). The vipers were conserved at −80°C until total RNA was extracted from the venom glands

2.2.1. Total RNA extraction. Total RNA was prepared from the venom glands as described by Chomczynski and Sacchi, [24] using Trizol reagent (Invitrogen BV, Leek, The Netherlands).

2.2.2. RT-PCR. Single-stranded cDNAs were prepared from total RNA (2 μg) using the First Stranded reaction kit (Amersham Biosciences, USA) and random hexamers (600 ng/ml). The forward primer: 5′-CAGAGGGAGCCTGGAGGTGCTTCTGGACCC-3′ and the reverse primer: 5′-TCAGTATTGCATTCAGAATAATAGAGTAAC-3′ were used to perform 30 cycles of PCR with 30 s of denaturation at 94°C, 30 s of annealing at 55°C and 1 min extension at 72°C.

2.2.3. Ligation and molecular cloning. The PCR products were ligated into the PCR 2.1 vector (Invitrogen) using the deoxythymidine-tailed method. Constructs with inserts were selected by PCR, using M13 vector primers.

2.2.4. Plasmidic DNA extraction. Standard techniques were used for plasmid extraction from *Escherichia coli* [25].

2.2.5. DNA sequencing. DNA sequencing was performed automatically by the Genopole, a technical platform in Institut Pasteur of Paris.

2.2.6. Sequence analysis. Single site mutations were verified on chromatograms before submitting the nucleotide and protein sequences to gene and protein databanks using Fasta and Blastn softwares. ClustalW (version 3.0) was used for alignments and Mac Molly software for translation and prediction of isoelectric points.

2.2.7. Molecular modeling. We assessed conformational and energetic consequences of the amino acid replacements using the high-resolution crystal structure of the non-covalent vipoxin complex (PDB code 1JLT, 1.4 Å resolution) as the template protein for modeling vaspin. Internal energy of the vaspin model was minimized followed by a 10 ns molecular dynamics trajectory in a continuum solvent with a dielectric function of 4r. Visualization and computations were performed using a SGI graphic station and the insight II software (Accelrys, San Diego, CA, USA).

3. Results

PLA_2 primers corresponding to a non-coding region of the PLA_2 gene were used to amplify a 570 bp DNA fragment from the total RNA. For each snake, 96 clones from two independent PCR experiments were sequenced in forward and reverse sense and the results were consistent. ClustalW identified five groups of DNA encoding PLA_2 sequences for which all members were identical. Two of these five groups were present in all *Vaa* snake glands (data not shown) whereas three of them were identified only in the glands of the vipers captured in southeastern France. They were very similar to neurotoxic PLA_2. Group 1 was 100% identical to the *Vamam* ammodytoxin B [21] and was thus an active neurotoxin, group 2, 95% identical to the *Daboia russelli siamensis* (formerly *Vipera russelli formosensis*) RV7; and group 3, 95.4% identical to the RV4 of the same species (Fig. 1) [21].

The theoretical p*I* of the group 2 PLA_2 was 4.2 and that of group 3, 8.7. The cDNAs for group 1, 2 and 3 PLA_2 each encode a signal peptide of 16 amino acid residues followed by a peptide chain of 122 residues. The deduced proteins of the group 2 (acidic) and 3 (basic) sequences, present high degrees of sequence identity to three distinct heterodimeric neurotoxins (see Fig. 2). They are very similar to the acidic and basic subunits respectively of PLA_2-I (98.75%), vipoxin (98.75%) and RV4/RV7 (92.00%) complexes [11,15,26,27]. The group 2 subunit is identical to the acidic subunit of PLA_2-I from *Vaz*, 98.3% identical to the vipoxin inhibitor (PDB code 1JLT, the most recently described vipoxin isoform sequence of the PLA_2, from *Vamme* venom) and only 92.7% to RV7. In common with the vipoxin inhibitor, His48, essential for catalysis, is replaced by a Gln [28], implying that the group 2 subunit lacks enzymatic activity. RV7 differs from the others by the conservation of the catalytic activity (His48) and eight other amino acid differences. The acidic subunits of *V. aspis* offspring differ from those of *V. ammodytes* and *Daboia* by a Thr70Met substitution. The group 3 protein is very similar to

Table 1
PLA_2 neurotoxins from European viper venom

Viper subspecies	Neurotoxin(s)	Activity	References
Vaa	none	–	–
Vaz	PLA_2-I	postsynaptic heterodimer associating a presynaptic basic subunit and a PLA_2-like subunit	[15,16]
Vamam	ammodytoxin A, B, C	presynaptic monomer	[8]
	vipoxin	postsynaptic heterodimer associating a presynaptic basic subunit and a PLA_2-like subunit	[9,12,13]
Vamme	vipoxin	postsynaptic heterodimer associating a presynaptic basic subunit and a PLA_2-like subunit	[10,11]
Vbb	none	–	–

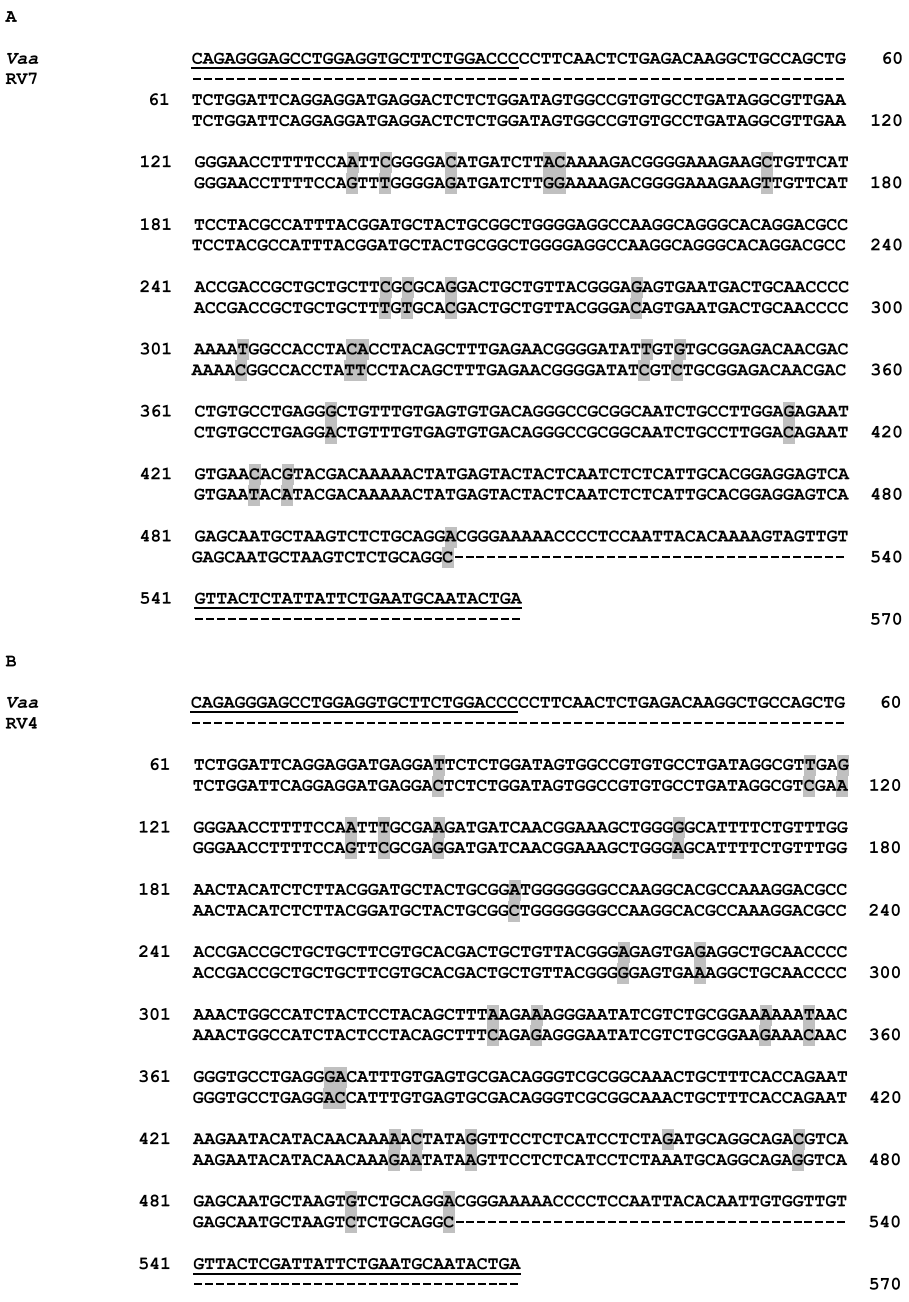

Fig. 1. Alignment of the cDNA nucleotide sequences of the group 2 and 3 *Vaa* PLA_2 with RV7 and RV4. The primers used for the PCR amplification of the cDNA are underlined. Mutations are shadowed.

the vipoxin (basic subunit), with a single Lys118Arg substitution: it presents 99.2% identity and is thus closer to vipoxin than to PLA_2-I in which there are Gln79Lys, Leu88Gln and Lys120Leu substitutions. It is thus likely that the group 2 and group 3 proteins are acidic and basic subunits forming a heterodimeric PLA_2, which we call vaspin.

The small amount of venom (10–20 μl) available did not allow us to purify vaspin either for crystallization or for biological tests. However, the high degree of sequence identity (98.75%) with vipoxin allowed us to use the crystal structure of vipoxin as a template to assess the consequences of the amino acid substitutions on the stability, structure and biological activity of the complex. Fig. 3 shows a molecular model of vaspin, highlighting the interface between the subunits.

Two amino acid substitutions exist in the acidic subunit of vaspin (Ala35Gly and Thr70Met). Gly35 is not exposed to the solvent and presents no steric hindrance. The Ca^{2+} binding loop that is located immediately upstream does not appear to be functionally or structurally altered. The Thr70Met replacement does not alter the electrostatic charge. Although the aliphatic side chain of Met70 is twice as long as that of Thr, it is not responsible for steric hindrance. A cavity opposite position 70, formed by residues Tyr28, Phe46, Asp49 and the Cys50–Cys133 disulfide bond of the basic subunit, is large enough to accommodate this side chain without atomic contacts. The new side chain would displace between one and three of the interfacial water molecules in 1JLT into the bulk solvent. This leads to the loss of one or two hydrogen bonds. This destabilizing enthalpic effect is countered by the

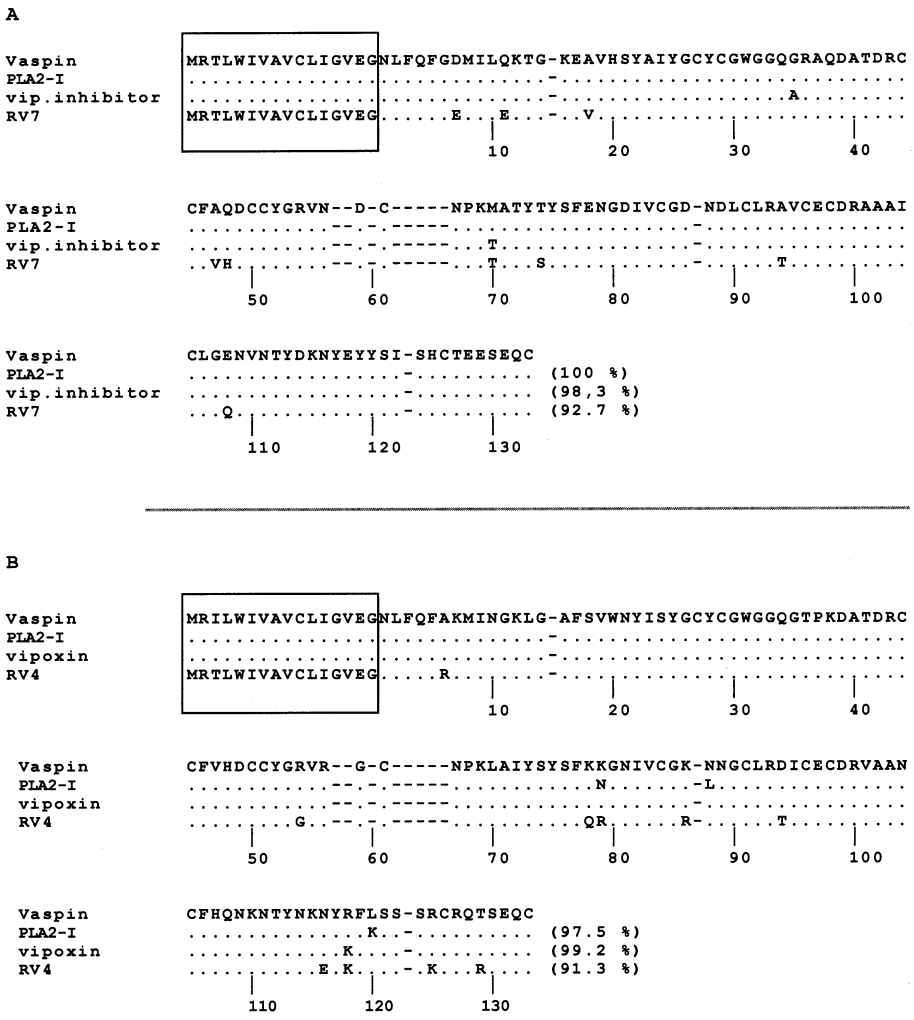

Fig. 2. Comparison of the predicted amino acid sequences for the acidic and basic subunits of the neurotoxin. Squares show the 16 residue long signal peptide. The common numbering of PLA_2s by Renetseder [34] is used. A dot indicates identical amino acid residue, and gaps introduced to optimize the alignment are shown by dashes. Numbers in parentheses are percentages of sequence identity to the first sequence. A: Alignment of acidic subunits of PLA_2 neurotoxins from: *Vaz* (PLA_2-I; gi: 1709547), *Vamme* (vipoxin inhibitor; gi: 16974941), *D. russelli siamensis* (RV4; gi: 64453). B: Alignment of basic subunits of PLA_2 neurotoxins from: *Vaz* (PLA_2-I; gi: 1709548), *Vamme* (vipoxin; gi: 16974940), *D. russelli siamensis* (RV7; gi: 64453).

positive entropy difference resulting from the release of the interface water molecules into the bulk solvent. In view of this entropy–enthalpy compensation effect of Thr70Met in the acidic subunit, we predict that the stability of the vaspin complex is similar to that of the vipoxin complex. The Met70 side chain moves between 4.1 and 7.4 Å away from that of the acidic subunit Asp49. Finally, as in the structure by Banumathi et al. [10], amino acids 35 and 70 are not involved in the interaction with the basic subunit.

Position 118 in the basic subunit is exposed to the solvent. As in the X-ray structure [10], it is not involved in the interaction with the acidic subunit [10] and has no effects on the conformation of the enzyme or of the complex. Furthermore, this substitution is not located within the regions involved in the neurotoxicity of postsynaptic neurotoxins like vipoxin, i.e. residues 6, 12, 76–81 (the β-wing) and 119–125 [26]. Moreover, this substitution is also found in the basic subunit of *Vaz* PLA_2-I (Lys120) without loss of neurotoxicity.

The energy minimization and molecular dynamics simulations result in a stable complex. The RMSDs of Cα distances between the model and the template structures over the simulation trajectory are of about 2 Å. In conclusion, the observed substitutions seem to have no substantial effect on the stability of the complex and cause only limited structural difference. It is thus likely that vaspin is an active neurotoxin and that it contributes to the manifestation of neurological symptoms.

4. Discussion

We report the first description of two neurotoxins in the venom of *Vaa* snakes from two departments of Southeastern France, following molecular cloning of the cDNA of venom gland total mRNA. One is a monomeric PLA_2, perfectly identical to ammodytoxin B found in the venom of *Vamam*, which acts presynaptically at the neuromuscular junction [27]. The other is a novel non-covalent PLA_2 heterodimer, named vaspin. The signal peptides of the three PLA_2 sequences contain the expected 'positive-hydrophobic-polar' domains required for secretion [29], and thus participation in the toxicity of

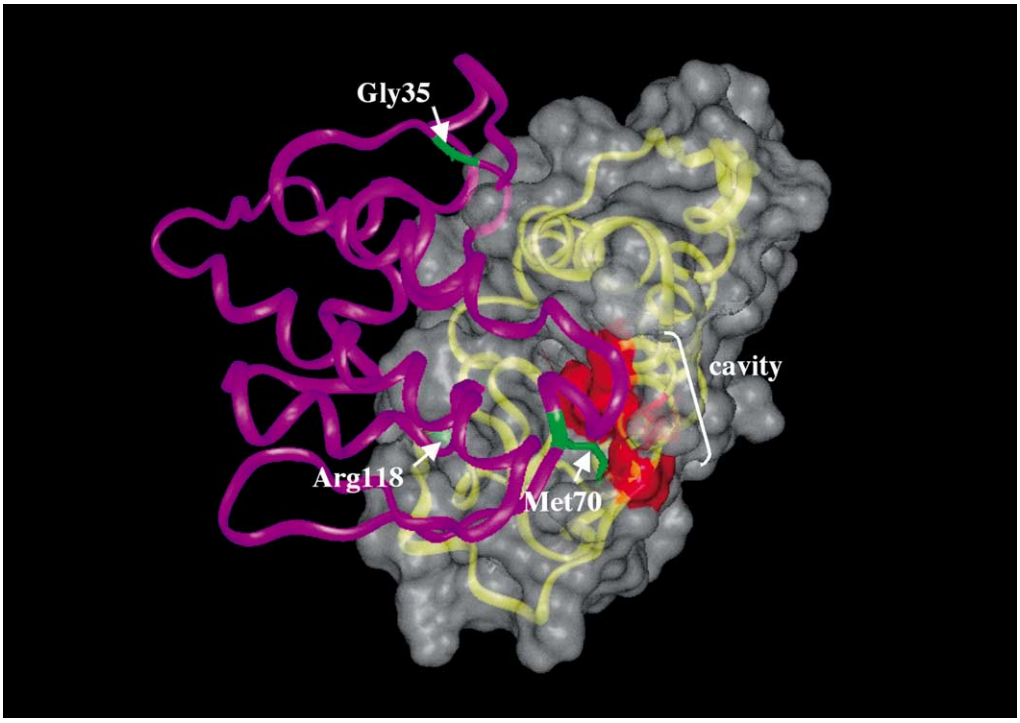

Fig. 3. A point in the dynamics trajectory of vaspin, the neurotoxic non-covalent PLA_2 heterodimer. The molecular model is derived from the crystallographic structure of the vipoxin complex (PDB id: 1JLT). The acidic subunit is in magenta ribbon and the basic one in yellow. Replaced amino acids are colored in green. The solvent accessible surface of the amino acids that form a cavity in front of Met70 is shadowed in red.

the venom. Vaspin is very similar to three known neurotoxic complexes: the postsynaptic PLA_2-I from *Vaz*, vipoxin from *Vamme* and the presynaptic RV4/RV7 complex from *D. russelli siamensis*. Blinov et al. (1979) have reported that the vipoxin complex had a specific effect on the postsynaptic membrane, decreasing its sensitivity to acetylcholine [30]. This is consistent with the inhibitory effect of PLA_2-I on the contractile response to direct stimulation of mouse diaphragm [31]. In view of the sequence and structural similarities, vaspin must display a similar activity to vipoxin and PLA_2-I, namely postsynaptic blocking of the neuromuscular junction.

Since neurological symptoms following *Vaa* envenomations had never been reported near Nice before 1992, the *Vaa* venom composition must have changed around that date. Although there is ambiguity on the occurrence of vipoxin in *ammodytes* subspecies, toxicity studies with *ammodytes* venoms after gel filtration never found both the heterodimeric and the monomeric neurotoxins in the same snake. The appearance in the same venom of these two neurotoxins probably results from two distinct mechanisms, which should be considered separately.

Firstly, the presence of ammodytoxin B could be due to the occasional expression of a preexisting *Vaa* gene under particular environmental or physiological conditions. Alternatively, this gene could come from a nearby Italian population of *Vamam* either by an expansion of the population or from interbreeding between *Vaa* and *Vamam* (possibly following accidental introduction of *Vamam*). Such hybrids can be produced in captivity and have been observed in Italy [32]. This is consistent with the unexpected neurological symptoms observed in Italy (between 1980 and 1984) after *Vaa* bites [33]. It should be noted that a perfect identity of the nucleotidic sequences was observed between ammodytoxin B-encoding mRNA of *Vaa* and *Vamam*. Since it is unlikely that preexisting genes have evolved in two distinct offsprings without developing any substitution, we therefore support the second hypothesis.

Secondly, vaspin is as similar to vipoxin as to PLA_2-I (98.75% identity). Gene flow for vaspin is unlikely because of the geographical barriers that separate the populations in which it is expressed from the *aspis* population near Nice. Banumathi et al. [10] stated that the vipoxin inhibitor 'is a product of divergent evolution of the unstable PLA_2' to 'stabilize it and in this way preserve the pharmacological activity and toxicity for a long period'. Our study suggests that this is not the case. Indeed, neurotoxic heterodimers are likely to be the product of the divergent evolution of a duplicated ancestral PLA_2 gene. As these similar complexes are found in different populations, the gene must have duplicated and the copies diverged before the separation of the *aspis* and *ammodytes* groups. Thereafter the PLA_2-encoding genes evolved to produce these different complexes. The sequence differences between the two PLA_2-subunits differ according to the species. The two genes may thus have evolved independently until the acquisition of the new function. On another hand, amino acid sequence similarities between the acidic and basic subunits of each vaspin (63%), vipoxin (62%) and PLA_2-I (65%) are less than those between the acidic subunit of vaspin and another PLA_2, ammodytin I2 from *Vamam* (74%). Since we detected the ammodytin I2 gene in the *Vaa* (sensus stricto) genome (personal communication), the acidic subunits of the three neurotoxins may share a common ancestor with ammodytin I2. The appearance of the acidic subunits may thus not be the result of a directed evolution but the product of ran-

dom evolution conferring an advantage and with selective acting on the two subunits. The chaperone function of the acidic subunit may be an acquired function allowing postsynaptic membranes of the neuromuscular junction to be targeted. Accordingly, the genes for the two subunits of vaspin should be present in the *V. aspis* genome. It is possible that the genes are normally expressed at a low level or are silent and that their expression is increased by stress. In such a case, we should attempt to observe other spots of vaspin expression in some *Vaa* populations or expression of similar complexes.

In conclusion, we demonstrate the recent appearance of a new geographical race of *Vaa* in southeast France, which expresses both monomeric and heterodimeric neurotoxins in its venom. These neurotoxins result from two distinct events. One is that the ammodytoxin B gene was acquired from *Vamam* either by gene flow from Italy after interbreeding with *Vaa* or by clandestine introduction in Southeast France. The other is that vaspin results from the occasional expression of preexisting *Vaa* genes. The acidic subunit of the complex may share a common ancestor with the ammodytin I2 gene, which may have duplicated before the separation of *aspis* and *ammodytes* offspring. Phylogenetic studies of viperid PLA_2 and genomic analysis of *Vaa* snakes are underway to further elucidate this issue.

Acknowledgements: Financial support was provided by Institut Pasteur (PTR). We are grateful to Y. Doljansky, O. Grosselet and A. Teynié for capturing the snakes and for carrying out the herpetological survey.

References

[1] Audebert, F., Sorkine, M. and Bon, C. (1992) Toxicon 30, 599–609.
[2] Radonic, V., Budimir, D., Bradaric, N., Luksic, B., Sapunar, D. and Vilovic, K. (1997) Mil. Med. 162, 179–182.
[3] Komori, Y., Nikai, T., Taniguchi, K., Masuda, K. and Sugihara, H. (1999) Biochemistry 38, 11796–11803.
[4] Calderón, L., Lomonte, B., Gutiérrez, J.M., Tarkowsky, A. and Hanson, L. (1993) Toxicon 31, 743–753.
[5] Komori, Y., Nikai, T. and Sugihara, H. (1993) Int. J. Biochem. 25, 761–767.
[6] Komori, Y. and Sugihara, H. (1988b) Toxicon 26, 1193–1203.
[7] Komori, Y. and Sugihara, H. (1990) Toxicon 28, 359–369.
[8] Ritonja, A. and Gubensek, F. (1985) Biochim. Biophys. Acta 828, 306–312.
[9] Mancheva, I., Kleinschmidt, T., Aleksiev, B. and Braunitzer, G. (1987) Biol. Chem. Hoppe-Seyler 368, 343–352.
[10] Banumathi, S., Rjashankar, K.R., Nötzel, C., Aleksiev, B., Singh Tej, P., Genov, N. and Betzel, C. (2001) Acta Crystallogr. D 57, 1552–1559.
[11] Perbandt, M., Wilson, J.C., Eschenburg, S., Mancheva, I., Aleksiev, B., Genov, N., Willingmann, P., Weber, W., Singh, T.P. and Betzel, C. (1997) FEBS Lett. 412, 573–577.
[12] Devedjiev, Y., Atanasov, B., Mancheva, I. and Aleksiev, B. (1993) J. Mol. Biol. 229, 1147–1149.
[13] Betzel, C., Visanji, M., Wilson, K.S., Genov, N., Mancheva, I., Aleksiev, B. and Singh, T. (1993) J. Mol. Biol. 231, 498–500.
[14] Devedjiev, Y., Popov, A., Atanasov, B. and Bartunik, H.D. (1997) J. Mol. Biol. 266, 160–172.
[15] Komori, Y., Masuda, K., Nikai, T. and Sugihara, H. (1996) Arch. Biochem. Biophys. 327, 303–307.
[16] Komori, Y., Nikai, T. and Sugihara, H. (1998) J. Nat. Toxins 7, 101–108.
[17] de Haro, L., Choumet, V., Robbe-Vincent, A., Bon, C., Jouglard, J. and Perringue, C. (1994) Rev. Prat. M.G. 265, 20–23.
[18] de Haro, L., Robbe-Vincent, A., Saliou, B., Valli, M., Bon, C. and Choumet, V. (2002) Hum. Exp. Toxicol. 21, 137–145.
[19] Saint-Girons, H., Duguy, R. and Detrait, J. (1983) Bull. Soc. Hist. Nat. Toulouse 119, 81–86.
[20] Chippaux, J.P., Williams, V. and White, J. (1991) Toxicon 49, 1279–1303.
[21] Kordis, D., Pungercar, J., Strukelj, B., Liang, N.S. and Gubensek, F. (1990) Nucleic Acids Res. 18, 4016.
[22] Sasa, M. (1999) Toxicon 37, 49–252.
[23] Ohno, M., Menez, R., Ogawa, T., Danse, J.-M., Shimohigashi, Y., Fromen, C., Ducancel, F., Zinn-Justin, S., Le Du, M.-H., Boulain, J.-C., Tamiya, T. and Ménez, A. (1998) Prog. Nucleic Acid Res. Mol. Biol. 59, 307–364.
[24] Chomczynski, P. and Sacchi, N. (1987) Anal. Biochem. 162, 156–159.
[25] Holmes, P.S. and Quigley, M. (1981) Anal. Biochem. 114, 187–193.
[26] Wang, Y.M., Lu, P.J., Ho, C.L. and Tsai, I.H. (1992) Eur. J. Biochem. 209, 635–641.
[27] Lee, C.Y., Tsai, M.C., Chen, Y.M., Ritonja, A. and Gubensek, F. (1984) Arch. Intern. Pharmacodyn. Thér. 268, 313–324.
[28] Betzel, C., Genov, N., Rajashankar, K.R. and Singh, T.P. (1999) Cell. Mol. Life Sci. 56, 384–397.
[29] Von Heijne, G. (1990) J. Membr. Biol. 115, 195–201.
[30] Blinov, N.O., Tchorbanov, B.P., Grishin, E.V. and Aleksiev, B.V. (1979) C.R. Acad. Bulg. Sci. 32, 663–666.
[31] Komori, Y., Nikai, T. and Sugihara, H. (1990) Comp. Biochem. Physiol. 97, 507–514.
[32] Naullau, G. (1997) in: La vipère aspic, Eveil nature, Angoulème.
[33] Beer, E. and Putorti, F. (1998) Toxicon 36, 697–701.
[34] Renetseder, R., Dijkstra, B.W., Huizinga, K., Kalk, K.H. and Drenth, J. (1988) J. Mol. Biol. 200, 181–188.