The Diversity of Bioactive Proteins in Australian Snake Venoms*

Geoff W. Birrell‡§¶, Stephen T. H. Earl‡§¶, Tristan P. Wallis‡‖, Paul P. Masci‡‡, John de Jersey§§, Jeffrey J. Gorman‡‖**, and Martin F. Lavin‡§¶¶**

Australian elapid snakes are among the most venomous in the world. Their venoms contain multiple components that target blood hemostasis, neuromuscular signaling, and the cardiovascular system. We describe here a comprehensive approach to separation and identification of the venom proteins from 18 of these snake species, representing nine genera. The venom protein components were separated by two-dimensional PAGE and identified using mass spectrometry and *de novo* peptide sequencing. The venoms are complex mixtures showing up to 200 protein spots varying in size from <7 to over 150 kDa and in pI from 3 to >10. These include many proteins identified previously in Australian snake venoms, homologs identified in other snake species, and some novel proteins. In many cases multiple trains of spots were typically observed in the higher molecular mass range (>20 kDa) (indicative of post-translational modification). Venom proteins and their post-translational modifications were characterized using specific antibodies, phosphoprotein- and glycoprotein-specific stains, enzymatic digestion, lectin binding, and antivenom reactivity. In the lower molecular weight range, several proteins were identified, but the predominant species were phospholipase A_2 and α-neurotoxins, both represented by different sequence variants. The higher molecular weight range contained proteases, nucleotidases, oxidases, and homologs of mammalian coagulation factors. This information together with the identification of several novel proteins (metalloproteinases, vespryns, phospholipase A_2 inhibitors, protein-disulfide isomerase, 5′-nucleotidases, cysteine-rich secreted proteins, C-type lectins, and acetylcholinesterases) aids in understanding the lethal mechanisms of elapid snake venoms and represents a valuable resource for future development of novel human therapeutics. ***Molecular & Cellular Proteomics 6:973–986, 2007.***

The venomous land snakes of Australia belong to the Elapidae family that also includes the Afro-Asian cobras, the American coral snakes, the Asian kraits, and the African mambas (1). The venoms are complex mixtures of proteins and peptides possessing a variety of biological activities, and because the amount of venom injected into an animal that reaches the bloodstream may be minute, venom components must achieve their effects at very low concentrations. The use of snake venom in different pathophysiological conditions has been mentioned in homeopathy and folk medicine for centuries. Recently there have been a number of examples of snake venoms used in the development of novel human therapeutics. These include the antihypertensive drug captopril (2), modeled from the venom of the Brazilian arrowhead viper (*Bothrops jaracusa*); the anticoagulant Integrilin (eptifibatide (3)), a heptapeptide derived from a protein found in the venom of the American southeastern pygmy rattlesnake (*Sistrurus miliarius barbouri*); Ancrod (4), a compound isolated from the venom of the Malaysian pit viper (*Agkistrodon rhodostoma*) for use in the treatment of heparin-induced thrombocytopenia and stroke; and alfimeprase, a novel fibrinolytic metalloproteinase for thrombolysis derived from southern copperhead snake (*Agkistrodon contortrix contortrix*) venom (5). Two venom proteins from the Australian brown snake, *Pseudonaja textilis*, are currently in development as human therapeutics (QRxPharma). The first is a single agent procoagulant that is a homolog of mammalian Factor Xa prothrombin activator (6), whereas the other is a plasmin inhibitor, named Textilinin-1, with antihemorrhagic properties (7).

Although there is much known about the protein compositions of venoms from Asian and American snakes, comparatively little is known of Australian snakes. This is despite the top 10 most toxic snakes (determined by LD_{50} (lethal dose in mice to kill 50%)) being Australian elapid snakes (8). Australian snakes are most closely related to Asian snakes, and although they have significantly higher toxicity, they cause far fewer deaths by envenomation than their Asian counterparts, most likely due to lower population density and more widespread availability of suitable health care in Australia (8). Proteins and peptides comprise the majority of the dry weight of elapid snake venoms, whereas other components include metallic cations, carbohydrates, nucleosides, biogenic amines, and low levels of free amino acids and lipids (9, 10). Venoms from Australian elapids can be loosely divided into procoagulant and anticoagulant types. The procoagulant venoms contain serine proteases (prothrombin activators) that cleave prothrombin to produce thrombin in the coagulation

From ‡The Queensland Institute of Medical Research, P. O. Royal Brisbane Hospital, Brisbane 4029, Australia and the §Central Clinical Division and ‡‡Southern Clinical Division, Faculty of Health Sciences, ‖Institute for Molecular Biosciences, and §§School of Molecular and Microbial Sciences, University of Queensland, Brisbane 4072, Australia

Received, November 1, 2006, and in revised form, February 1, 2007
Published, MCP Papers in Press, February 21, 2007, DOI 10.1074/mcp.M600419-MCP200

cascade resulting in coagulation. Snake venom prothrombin activators are classified into four groups based on functional characteristics, structural properties, and cofactor requirements (11). Group A and B prothrombin activators are metalloproteinases, whereas Group C and D prothrombin activators are serine proteinases. Some Australian snake venoms are known to contain Group C or Group D prothrombin activators (12). Group C prothrombin activators resemble the mammalian Factor Xa (FXa)[1]-Factor Va (FVa) complex, whereas Group D activators are structurally and functionally similar to FXa alone (12, 13). The Group C prothrombin activator from the Australian brown snake, *P. textilis*, has been extensively studied and named Pseutarin C (14). The 1461-amino acid FVa-like non-enzymatic subunit of Pseutarin C has been shown to share 50% sequence identity and significant structural homology with human FVa (15). Another hemostasis-related family of proteins identified in Australian snake venom is the textilinin family of plasmin inhibitors (16). These 7-kDa proteins share ~45% identity with aprotinin, a bovine Kunitz-type serine protease inhibitor that acts on plasmin and kallikrein to reduce blood loss during surgery. Six isoforms of textilinin have been identified in *P. textilis* venom gland-derived cDNA (7).

Australian snake venoms are also known to contain PLA_2s and peptidic neurotoxins (for a review, see Ref. 17). Australian elapid PLA_2s have seven conserved disulfide bonds and molecular masses of 13–15 kDa. As well as phospholipase activity, individual PLA_2s are known to have myotoxic, neurotoxic, and/or anticoagulant activities. The α-neurotoxins found in Australian elapids are postsynaptic blocking short or long chain neurotoxins (18, 19). Short and long chain neurotoxins have similar effects and bind with high affinity to skeletal nicotinic acetylcholine receptors. The short chain neurotoxins have four disulfide bonds and are ~60 amino acids in length, whereas the long chain neurotoxins have five disulfide bonds and are ~73 amino acids in length. More recently, neurotoxic cysteine-rich secreted proteins (CRISPs) have been identified and characterized from the Australian elapids *Pseudechis australis* and *Pseudechis porphyriacus* (20, 21). These 211-amino acid proteins show the ability to block cyclic nucleotide-gated ion channels involved in signaling from the visual and olfactory systems.

Other proteins known to exist in Australian elapid venoms include L-amino-acid oxidase, nerve growth factor (NGF), and natriuretic peptides. L-Amino-acid oxidase was identified in *P. australis* venom and in purified form was found to be antibacterial when tested against the important pathogen *Aeromonas hydrophila* (22). This protein has also been identified in Viperidae snake venoms. NGF has been identified in multiple Asian and African snake venoms but has been reported only recently as a component of Australian elapid venoms (23). NGF from *Oxyuranus scutellatus* venom is glycosylated and shows the ability to induce neurite outgrowth of PC12 cells, a standard assay for NGF activity (23). Natriuretic peptides from *Oxyuranus microlepidotus* venom show potent arterial smooth muscle relaxant effects (24). Natriuretic peptides have been identified and characterized from several Australian elapid venoms and represent the smallest known proteins from these venoms at only 35–39 amino acids (25).

The present study describes a thorough screening and identification of the venom proteins present in 18 Australian elapid snake species representing nine genera of the most venomous snakes on earth. In addition, post-translational modifications such as glycosylation, phosphorylation, and γ-carboxylation were examined in the venom proteins using specific stains and antibodies. Glycoforms of multiple proteins were identified and characterized using lectin binding specificity. Also antivenom reactivity of venom proteins was examined using tiger snake (*Notechis*) antivenom raised in horses for clinical use in envenomated people. Many proteins previously unknown in Australian snake venoms were identified based on sequence matches to venom proteins from other snakes. This information sheds light on evolutionary relationships between the different snake species and the clinical manifestations of envenomation. This comprehensive proteomics analysis brings Australian elapid snakes in line with their well studied American and Asian counterparts and represents a valuable resource for the future development of novel human therapeutics.

EXPERIMENTAL PROCEDURES

Materials: Snake Venoms and Chemicals—Venom samples from the 18 snake species (Table I) were obtained in lyophilized form from Venom Supplies Pty. Ltd. (Tanunda, South Australia, Australia). Venom samples were collected over the course of a year and pooled from at least 10 snakes from each species to reduce seasonal and individual variation. Lyophilized venom samples were resuspended in 50% glycerol, 50% PBS to a final protein concentration of 10 mg/ml. Water was prepared using a Milli-Q system (Millipore, Bedford, MA). FITC-labeled lectins were from Vector Laboratories (Burlingame, CA). PNGase F and phosphatase (Antarctic phosphatase) were from New England Biolabs (Beverly, MA). Antiserum to *P. textilis* Factor Xa-like heavy chain antisera was raised in sheep against the purified protein (26). Monoclonal antibody against γ-carboxyglutamate (Gla) residues was from American Diagnostica Inc. (Greenwich, CT). Tiger snake (*Notechis scutatus*) antivenom was from Commonwealth Serum Laboratories Ltd. (Victoria, Australia). All other chemicals were from Sigma unless stated otherwise.

Electrophoresis—Prior to the first dimension IEF, 30 μl (300 μg total) of reconstituted venom was added to 170 μl of rehydration

[1] The abbreviations used are: FXa, Factor Xa; FVa, Factor Va; 2D, two-dimensional; 1D, one-dimensional; Gla, γ-carboxyglutamate; PNGase F, peptidyl-*N*-glycosidase F; ConA, concanavalin A; WGA, wheat germ agglutinin; RCA120, *Ricinus communis* agglutinin 120; GalNAc, *N*-acetylgalactosamine; GlcNAc, *N*-acetylglucosamine; PLA_2, phospholipase A_2; NGF, nerve growth factor; CRISP, cysteine-rich secreted protein; PDI, protein-disulfide isomerase; SVMP, snake venom metalloproteinase; GRP78, glucose-regulated protein 78; ACTH, adrenocorticotropic hormone; clip, corticotropin-like intermediate lobe peptide; PMM, peptide mass mapping; SNA, *Sambucus nigra* lectin; UEA, *Ulex europaeus* agglutinin.

solution containing 7 M urea, 2 M thiourea, 4% (w/v) CHAPS, 10 mM DTT, and 0.2% (v/v) pH 3–10 Biolyte ampholytes (Bio-Rad). The final volume of 200 μl was applied to a precast Bio-Rad 11-cm pH 3–10 linear IPG strip. First dimension IEF was carried out in a Bio-Rad Protean isoelectric focusing unit at 20 °C using a three-phase program: 250-V rapid gradient for 15 min, 250–8000-V linear gradient for 3 h, and 8000-V rapid gradient to a total of 40,000 V-h. Prior to the second dimension, the proteins in the IPG strips were reduced and alkylated by sequential 15-min incubations in a solution containing 0.05 M Tris-HCl, pH 8.8, 6 M urea, 2% (w/v) SDS, 20% (v/v) glycerol, and (i) 10 mM DTT in 100 mM ammonium bicarbonate for reduction and (ii) 55 mM iodoacetamide in 100 mM ammonium bicarbonate for alkylation. IPG strips were then applied to 12% Tris-HCl acrylamide gels (Bio-Rad Criterion, 13 × 10 cm) for second dimension electrophoresis at 200 V for 65 min. Gels were stained with a mass spectrometry-compatible silver stain based on a method described previously (27).

Identification of Venom Components and de Novo Sequencing Using MALDI-TOF Mass Spectrometry—Protein spots from silver-stained 2D PAGE were excised, washed in water, and destained as described previously (28). Trypsin was added, and proteins were allowed to digest overnight at 37 °C prior to extraction. Extracted peptides were dried; resuspended in 50% ACN, 0.1% TFA; mixed 1:1 with matrix (10 mg/ml α-cyano-4-hydroxycinamic acid in 60% acetonitrile, 25 mM ammonium bicarbonate); and spotted on a MALDI plate. Peptides were analyzed by MALDI-TOF/TOF MS/MS using a 4700 Proteomics Analyzer (Applied Biosystems) operated in positive ion reflectron mode. MS data were acquired using 2000 shots of a neodymium-doped yttrium aluminium garnet (Nd:YAG) laser at 355 nm with a 200-Hz repetition rate and fixed intensity. MS data were calibrated via a plate wide external calibration using the 4700 Mass Standards kit (Applied Biosystems) containing des-Arg-bradykinin (MH^+ 904.458), angiotensin I (MH^+ 1296.685), Glu-fibrinopeptide B (MH^+ 1570.677), ACTH (1–17 clip, MH^+ 2093.087), ACTH (18–39 clip, MH^+ 2465.199), and ACTH (7–38 clip, MH^+ 3657.929). The top 50 most intense peptides detected for each spot in the MS mode were automatically selected for MS/MS analysis using 3000 laser shots at a fixed intensity ~20% greater than that used for MS. MS/MS data were calibrated against the MS/MS fragments of the $m/z = 1296.685$ angiotensin I peptide in the standards. With regard to the MS/MS analysis, no CID was used. Metastable decay was used exclusively to obtain fragment ion information without the introduction of CID gas into source 2. Interference from metastable decay of the precursor ions postsource 2 was precluded using metastable ion suppression

Alternatively peptides were analyzed using a Microflex MALDI-TOF PSD instrument (Bruker Daltonics, Bremen, Germany) operated in positive ion reflectron mode. MS data were acquired using 350 shots of a nitrogen laser at 355 nm with a 20-Hz repetition rate and varying intensity. MS data were calibrated via close external calibration using peptide standards (New England Biolabs) containing angiotensin I (MH^+ 1296.69), neurotensin (MH^+ 1672.92), ACTH (1–17 clip, MH^+ 2093.09), ACTH (18–39 clip, MH^+ 2465.20), and ACTH (7–38 clip, MH^+ 3657.93). The top three most intense peptides detected for each spot in the MS mode were automatically selected for PSD analysis using ~15 segments with 200 shots per segment. Initial laser power was ~25% greater than for MS. The segments were stitched together by Flex Analysis software (Version 2.4, Bruker Daltonics) for sequence interpretation. PSD data were internally calibrated.

MALDI-TOF/TOF MS/MS data from the 4700 Proteomics Analyzer were automatically analyzed using the GPS Explorer suite of software (Version 3.5 Build 321, Applied Biosystems). For each spot a combined MS and MS/MS analysis was performed in-house using a Mascot search engine (Version 1.9) and the Celera Discovery System database (CDS Combined KBMS2.1.20030813 containing 1,335,729 sequences, dated May 5, 2006). MS peptide tolerance was 100 ppm, and MS/MS tolerance was 0.3 Da. MALDI-TOF PSD data from the Microflex were automatically analyzed using Biotools 3.0 software (Bruker Daltonics). For each spot MS and PSD data were separately searched in-house using the Mascot search engine (Version 1.9) and the Chordata taxonomic subset of the National Center for Biotechnology Information non-redundant database (NCBInr 20060111; Chordata subset; 627,293 sequences; dated January 11, 2006). In both cases, mass tolerance was set at 150 ppm. All searches took into account carbamidomethylated cysteine and oxidized methionine. For the purposes of protein identification, no other post-translational modifications were considered, and the top matched protein from the database searches was included in Supplemental Table 1. The scores used to evaluate the quality of matches for MS/MS data appear in Supplemental Table 1 in columns titled "Ion Score" and "Ion Score confidence interval" calculated by the Mascot search engine. Protein and ion scores increase where the experimental fragmentation data matches theoretical fragmentation data. With regard to acceptance criteria for protein identification, those candidates with Mascot scores greater than the 95% confidence threshold (generally a protein score of 65) and whose protein mass and pI correlated with the 2D PAGE spot were automatically accepted. Those candidates with Mascot scores below the 95% confidence threshold but whose identity matched to a known snake venom protein were also included. Candidates still unknown were selected for *de novo* peptide sequencing.

For *de novo* peptide sequencing, MALDI-TOF/TOF MS/MS data were opened in Data Explorer (Applied Biosystems, Version 4.2) and deisotoped, and raw text peak lists were exported. The peak lists were analyzed using the automatic *de novo* function of PEAKS Studio software Version 2.4 (29) (Bioinformatics Solutions Inc., Ontario, Canada). Peptide sequences of 6 or more amino acids with a 100% confidence call using parent ion and fragment mass error tolerances of 0.1 Da were collected and matched to the NCBI non-redundant protein database using the protein Basic Local Alignment Search Tool (BLAST) algorithm (Version 2.2.14, www.ncbi.nlm.nih.gov/BLAST/).

Enzymatic Deglycosylation—Venom samples (50 μg) were precipitated in 8:1 acetone:methanol for 16 h at −20 °C. Samples were centrifuged for 30 min at 16000 × *g* and 4 °C to pellet protein. The supernatants were removed and discarded, and the protein pellets were allowed to air dry for 10 min under ambient conditions. PNGase F (New England Biolabs) was subsequently used according to the manufacturer's instructions. Reactions were allowed to proceed for 16 h at 37 °C.

Lectin Binding Specificity—Venom samples (100 μg) were separated by 1D PAGE as described above and transferred onto nitrocellulose membrane in Towbin buffer at 100 V for 1 h. Glycosylation profiles of the venom proteins were examined using a panel of eight lectins labeled with FITC (Fluorescein Lectin Kit 1, Vector Laboratories) as described previously (30).

Phosphoprotein and Glycoprotein Staining—Detection of phosphoproteins and glycoproteins in 1D PAGE was performed using the ProQ Diamond and ProQ Emerald fluorescent reagents, respectively (Molecular Probes, Invitrogen) according to the manufacturer's instructions. ProQ Emerald is a fluorescent form of the periodic acid-Schiff stain for carbohydrates that has a broad range of specificity for glycol containing molecules.

Immunoblotting and Antivenom Reactivity—Immunoblotting for Gla residues and the heavy chain of the FXa-like protease was performed as described previously (31). Tiger snake antivenom reactivity was performed similarly to immunoblotting as follows. Proteins were separated by 1D SDS-PAGE, transferred to nitrocellulose membrane, blocked in 5% skim milk, and incubated overnight in a 1:2000 dilution of antivenom in PBS with 0.05% Tween 20. After extensive washing, the membrane was incubated in a 1:2000 dilution of goat anti-horse

TABLE I
Snake venoms investigated

Genus	No.	Species	Common name
Notechis	1	*N. scutatus*	Mainland tiger
(tiger snakes)	2	*N. ater niger*	Revesby Island tiger
	3	*N. ater serventyi*	Chappell Island tiger
Pseudonaja	4	*P. nuchalis*	Westerm brown
(brown snakes)	5	*P. affinis*	Dugite
	6	*P. inframacula*	Peninsula brown
Austrelaps	7	*A. superbus*	Lowland copperhead
(copperheads)	8	*A. ramsayi*	Highland copperhead
Pseudechis	9	*P. porphyriacus*	Red-bellied black
(black snakes)	10	*P. australis*	King brown
	11	*P. guttatus*	Blue-bellied black
	12	*P. colletti*	Colletts snake
Acanthophis (death adder)	13	*A. antarcticus*	Common death adder
Oxyuranus (taipans)	14	*O. scutellatus*	Coastal taipan
	15	*O. microlepidotus*	Inland taipan
Tropidechis	16	*T. carinatus*	Rough scale snake
Rhinoplocephalus	17	*R. nigrescens*	Small-eyed snake
Hoplocephalus	18	*H. stephensii*	Stephen's banded snake

Ig conjugated to horseradish peroxidase, and signal was detected using ECL reagent.

RESULTS

Separation and Comparative Analysis of Venom Proteins from 18 Australian Elapid Snake Species

Protein components of the venoms from the 18 species listed in Table I were initially separated by 1D SDS-PAGE and subsequently silver-stained (Fig. 1). A similarity of protein bands between individual species within a single genus was apparent. For example, a prominent band of 30–35 kDa corresponding in size to the heavy chain of FXa-like protease was observed in several venoms including the *Notechis* (*lanes 1–3*), *Pseudonaja* (*lanes 4–6*), and *Oxyuranus* (*lanes 14* and *15*) species. In addition, several venoms showed significant staining of proteins primarily between 13 and 15 kDa, which is consistent with the size of PLA_2 isoforms known to be abundant in elapid snake venoms. Moreover the multiplicity of bands between 10 and 170 kDa for all species highlights the complexity of these venoms and necessitated the use of 2D PAGE to achieve resolution of individual protein isoforms.

The results in Fig. 2 show representative 2D PAGE silver-stained maps for a single species from all nine genera with resolution of proteins in the size range of 6–160 kDa over a pI range of 3–10. Between 100 and 200 individual protein spots were resolved in the different maps. *Horizontal trains* of spots are apparent in the *upper region* of the 2D PAGE maps consistent with multiple isoforms of individual proteins. This phenomenon has been observed previously in 2D PAGE of venom proteins from other snake species (32, 33). Although up to 200 spots were resolved on each gel, only a limited number of protein families appeared to be present. The 2D PAGE maps for all 18 species *without* annotation appear in

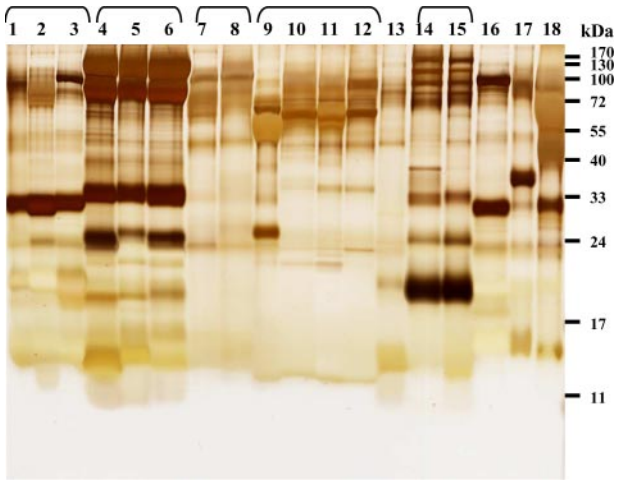

FIG. 1. **SDS-PAGE of 18 Australian snake venoms.** 100-μg samples of 18 crude venoms from the snakes listed in Table I were separated by 12% SDS-PAGE and subsequently silver-stained. *Brackets above lanes* indicate species of the same genus. *Lane 1*, *N. scutatus*; *lane 2*, *N. ater niger*; *lane 3*, *N. ater serventyi*; *lane 4*, *P. nuchalis*; *lane 5*, *P. affinis*; *lane 6*, *Pseudonaja inframacula*; *lane 7*, *A. superbus*; *lane 8*, *Austrelaps ramsayi*; *lane 9*, *P. porphyriacus*; *lane 10*, *P. australis*; *lane 11*, *P. guttatus*; *lane 12*, *P. colletti*; *lane 13*, *A. antarcticus*; *lane 14*, *O. scutellatus*; *lane 15*, *O. microlepidotus*; *lane 16*, *T. carinatus*; *lane 17*, *R. nigrescens*; *lane 18*, *H. stephensii*.

Supplemental Fig. 1, A-R, and with annotation in Supplemental Fig. 2, A-R. The mass spectrometry data corresponding to each identified protein spot appear in Supplemental Table 1. Comparison of the distinct protein spots on the maps showed features characteristic of species within the same genera. For example, comparison of the four *Pseudechis* species showed a high degree of similarity between *P. australis*, *Pseudechis guttatus*, and *Pseudechis colletti*. Although most of the protein families present in these three species also appear to be represented in the fourth member of this group, *P. porphyriacus*, it is evident that the protein pI values have a different pattern of distribution across the range of separation. It is also notable that *P. porphyriacus* is the only member of this genus known to have procoagulant activity, and the data described here point to additional variation at the protein level (8).

Identification of Proteins by Mass Spectrometry

Approximately 60 spots were selected from 2D PAGE for each species across a range of molecular sizes and pI values for further analysis. Gel spots were excised and digested with trypsin, and peptides were collected for analysis by MALDI-TOF, MALDI-TOF PSD, and MALDI-TOF/TOF MS/MS. The mass spectra were matched to the NCBI non-redundant or Celera databases to identify the protein spots using the Mascot search engine. Spectra for those spots that were not identified by this approach were subjected to *de novo* peptide sequence analysis using the PEAKS software package (Bioinformatics Solutions Inc.). The *de novo* peptide sequences were used in homology searches using the NCBI protein

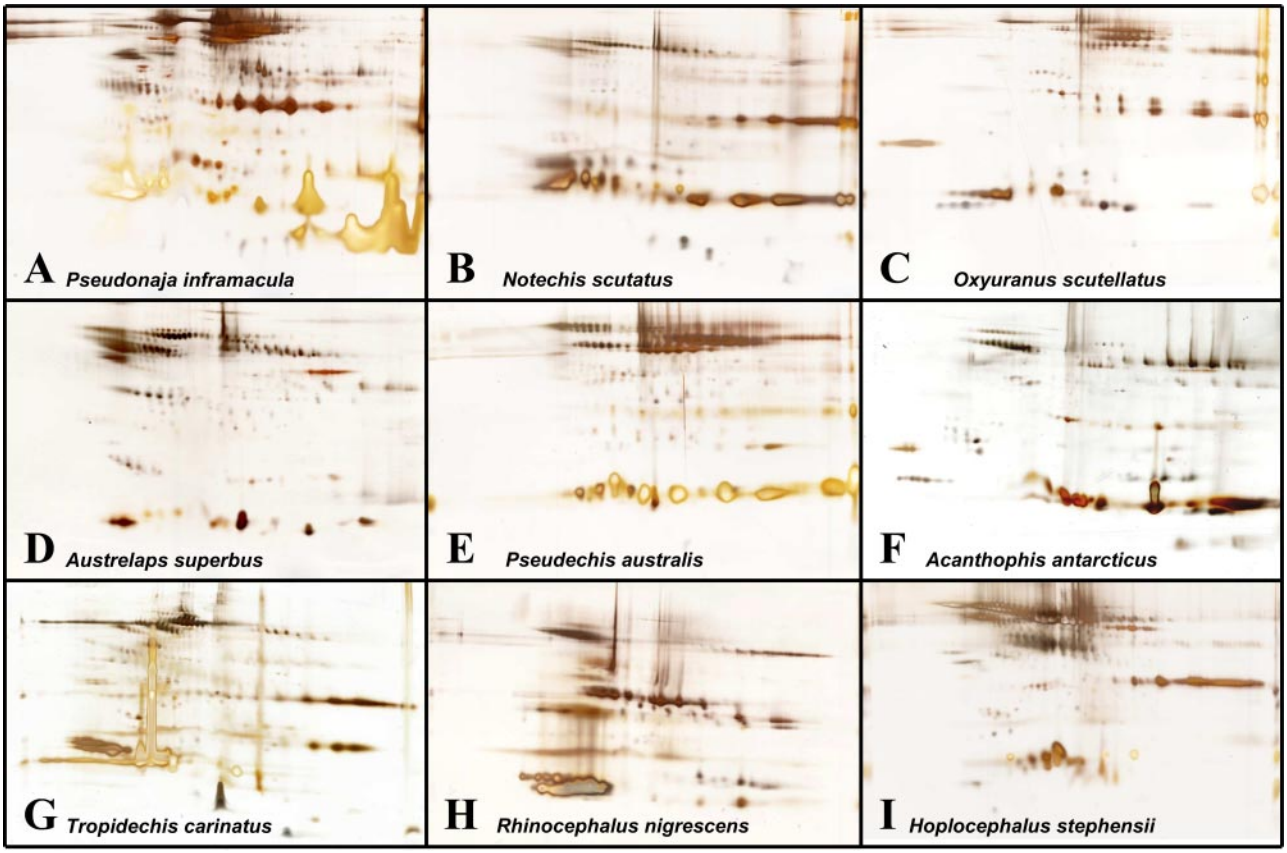

FIG. 2. **2D SDS-PAGE of crude venom from species representing all nine genera.** *A–I*, 300-μg samples of crude venoms representing nine species of different genera were subjected to isoelectric focusing on 11-cm pH 3–10 IEF strips and subsequently separated by 12% SDS-PAGE and silver-stained. Protein spots were excised for identification by MALDI-TOF/TOF MS/MS, MALDI-TOF PSD, and *de novo* peptide sequencing. Complete data are contained in Supplemental Figs. 1 and 2 and Supplemental Table 1.

BLAST facility (www.ncbi.nlm.nih.gov/BLAST/). Using this combined approach, between 24 and 51 spots for each species were confidently identified, representing a total of 17 different protein families (Table II). A number of protein spots were not identified using the above approach possibly because the peptide sequences derived were not sufficiently homologous to those in the sequence databases searched.

A total of 724 protein identifications were made, and a complete list of these together with their protein and ion scores appears in Supplemental Table 1. Various combinations of the different protein families were present in the 18 species within the nine genera studied (Table II). Supplemental Fig. 3 contains annotated fragmentation spectra for those 131 of 724 (18.1%) protein identifications based on a single peptide. Supplemental Fig. 4 contains annotated MALDI-TOF spectra for the 34 of 724 (4.7%) protein identifications based on peptide mass mapping (PMM). In some of the PMM spectra several major peaks were not matched suggesting either more than one protein in the spot or incomplete homology with database entries. Although a range of protein and ion scores were obtained in the mass spectrometry analysis, overall the data provided a high level of confidence that the identifications were correct. This was evident by the generally high protein and ion scores for the PMM and MS/MS data as well as high levels of sequence homology in the protein BLAST results from *de novo* peptide sequencing where the former approach failed to identify the protein. Identities and characteristics of the 17 protein families are provided below.

PLA_2s and PLA_2 Inhibitors—The most frequently encountered proteins identified across the species were PLA_2s that were found in all 18 venoms. In all cases there were multiple PLA_2 isoforms representing sequence variants. Because of the large number of isoforms and associated activities for these proteins, information on the specific PLA_2s identified within the different species can be accessed through the NCBI accession numbers provided in Supplemental Table 1. In the majority of cases the PLA_2 isoforms were in the molecular size range of 13–17 kDa as predicted from published data and in some cases their cDNA sequences (34). However, in other cases (for example *Acanthophis*, Fig. 2*F*) there was considerable size variation with PLA_2s ranging from 13 to 30 kDa. Surprisingly we also detected a protein homologous to the α isoform of PLA_2 inhibitor (35) (NCBI accession number CAB56615) in both *Pseudonaja nuchalis* and *O. microlepidotus*. This is the first description of a PLA_2 inhibitor in the

TABLE II
Protein families identified in the 18 snake venoms

Neuro, neurotoxin; Kunitz, Kunitz-like serine protease inhibitor; Inhib, inhibitor; LAAO, L-amino-acid oxidase; AChE, acetylcholinesterase; 5′Nuc, 5′-nucleotidase.

		Short Neuro	Long Neuro	Kunitz	Vespryn	PLA_2	PLA_2 Inhib	NGF	C-type lectin	FXa-like	CRISP	PDI	LAAO	AChE	SVMP	GRP78	5` Nuc	FVa-like
	Protein family number	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**	**17**
	Species																	
1	*N. scutatus*		+	+		+				+			+	+				
2	*N. ater niger*		+	+		+				+			+	+	+		+	
3	*N. ater serventyi*		+	+		+		+		+			+			+	+	
4	*P. nuchalis*		+	+		+	+	+	+	+		+						+
5	*P. affinis*	+	+	+		+			+	+								+
6	*P. inframacula*	+	+	+		+			+	+	+							+
7	*A. superbus*		+			+			+				+	+				
8	*A. ramsayi*					+					+		+					
9	*P. porphyriacus*			+		+		+		+	+				+			
10	*P. australis*		+	+		+		+					+		+			
11	*P. guttatus*			+		+		+					+		+		+	
12	*P. colletti*			+		+		+					+				+	
13	*A. antarcticus*		+	+		+		+	+				+	+	+			
14	*O. scutellatus*			+		+		+		+							+	+
15	*O. microlepidotus*	+	+			+	+	+		+							+	+
16	*T. carinatus*		+	+	+	+		+		+			+	+				
17	*R. nigrescens*					+		+		+			+		+			
18	*H. stephensii*					+		+	+	+	+		+		+		+	

venom of any snake. Previous reports have demonstrated that PLA_2 inhibitors are present in the blood of Elapidae, Hydrophidae, Boidae, and Colubridae genera, and it was assumed that they were present to provide protection against any PLA_2s appearing inadvertently in the blood (for a review, see Ref. 36).

Neurotoxins and Protease Inhibitors—Australian snake venoms are known to contain a multitude of neurotoxins including short and long chain α-neurotoxins and neurotoxic PLA_2s (8). Long chain neurotoxins were found in the majority of venoms. These were readily identifiable in all species of *Notechis* and *Pseudonaja* but only in one (*P. australis*) of the *Pseudechis* species. On the other hand, short chain neurotoxins were only detected in three species (Table II). The Kunitz-type serine protease inhibitor textilinin was identified in all species of the *Pseudonaja* genus as expected. We have previously identified multiple forms of textilinin both at the protein and cDNA level in *P. textilis* (7, 16). Proteins with homology to textilinin were also found in the *Notechis*, *Acanthophis*, *Pseudechis*, *Tropidechis*, and *Oxyuranus* genera. This molecule is an antifibrinolytic agent that inhibits plasmin (7).

Prothrombin Activators—Another snake protein known to affect hemostasis is the FXa-like prothrombin activator previously identified in *Pseudonaja* and other genera (37). The heavy chain of this protein was identified in all the *Notechis*, *Oxyuranus*, and *Pseudonaja* species along with *Tropidechis carinatus*, *Hoplocephalus stephensii*, *Rhinoplocephalus nigrescens*, and *P. porphyriacus*. This is consistent with the presence of procoagulant activity in all of these snakes (8). The heavy chain was identified as a train of spots at ~35 kDa and with pI generally ranging from 6 to 9. This peptide was not found in the *Austrelaps* or other three *Pseudechis* species or in *Acanthophis antarcticus*. This is in accordance with previous data showing that these snakes possess largely anticoagulant venoms (8). In addition, the light chain of the FXa-like molecule is known to contain Gla residues, and immunoblotting with Gla-specific antibody (Fig. 3*A*) showed reactivity only to species where the heavy chain was also identified by 2D PAGE and MS. It is also notable that the light chain was only identified by mass spectrometry in three species. This is in agreement with previous data where it has been difficult to detect the presence of this peptide by both Coomassie Blue and silver staining (31). The non-enzymatic cofactor of the prothrombin activator, FVa-like protein, was identified only in *Pseudonaja* and *Oxyuranus*, the only two genera known to contain a complete prothrombin activator (15, 37).

Vespryns—Using MS-based peptide sequencing we identified two peptides in a *T. carinatus* 2D PAGE protein spot (Supplemental Fig. 2P, spot 14) that showed homology to Ohanin, recently described for the first time in *Ophiophagus hannah* (king cobra) venom (38). Ohanin is the first of a new family of proteins named as vespryns. The peptides identified in *T. carinatus* covered 21 of the 109 residues (19%) of the mature *O. hannah* protein, and both peptides were located near the center of the protein. Primers were designed based on the full-length *O. hannah* mRNA sequence (NCBI acces-

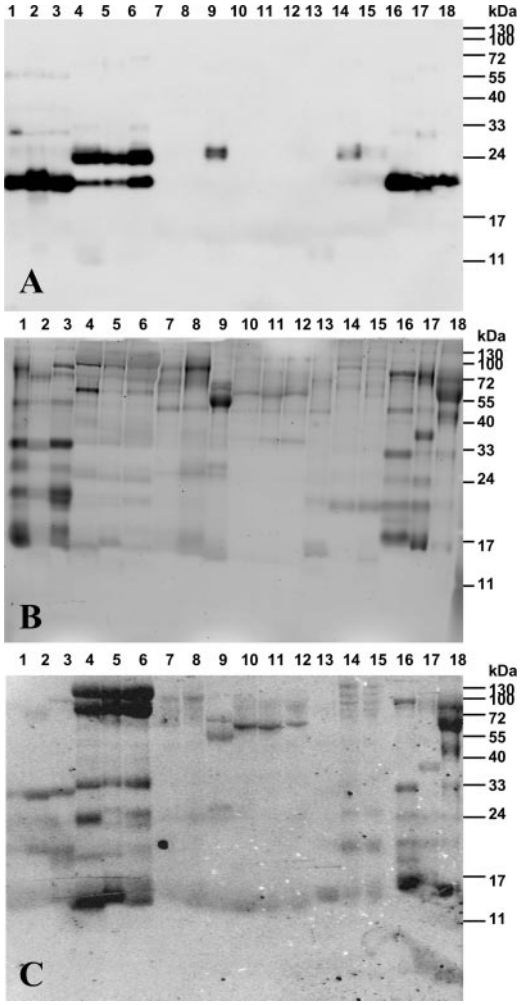

FIG. 3. **Post-translational modifications observed in the 18 venoms.** *A*, 100 μg of crude venoms were separated by 12% SDS-PAGE, transferred to nitrocellulose, and immunoblotted using antibody to Gla residues. *B* and *C*, 100 μg of crude venoms were separated by 12% SDS-PAGE, and the gels were subsequently stained with ProQ Diamond phosphoprotein-specific stain (*B*) or with ProQ Emerald glycoprotein-specific stain (*C*).

sion number AY351433) and were used to amplify the full-length cDNA from venom gland RNA from *T. carinatus* and several of the other species.[2] The presence of RNA transcripts in elapid venom glands other than *T. carinatus* suggests the presence of the protein in these venoms; however, no other protein spots in any of the other 17 venoms were identified as vespryns.

Metalloproteinases—In the higher molecular size region, seven of the 18 2D PAGE maps (39%), proteins were matched via MS to proteins homologous to snake venom metalloproteinases (SVMPs). These proteins have been observed in abundance in venoms of viper snakes and also in some elapids (39, 40). The peptides observed identified the Australian elapid metalloproteinases as PIII type SVMPs that contain a reprolysin zinc metalloprotease domain, a disintegrin domain, and a C-terminal cysteine-rich domain.

C-type Lectins—Protein spots of ~15 kDa from six of the 2D PAGE maps were matched via MS to a C-type lectin from *Bungarus* sp. venom. C-type lectins are non-enzymatic, calcium-dependent, sugar-binding proteins and have been identified previously in several Viperidae and a limited number of Elapidae venoms.

Other Protein Families—Acetylcholinesterase, NGF, 5′-nucleotidase, CRISP, L-amino-acid oxidase, protein-disulfide isomerase, and glucose-regulated protein 78 (GRP78) were also isolated in 2D PAGE maps of some of the venoms. Acetylcholinesterase has been characterized previously from *Bungarus fasciatus* venom (41) and in this study matched to a 70-kDa series of spots spanning ~2 pI units in five venoms (*A. antarcticus*, *Notechis ater niger*, *N. scutatus*, *Austrelaps superbus*, and *P. australis*). NGF has been identified recently as a component of six Australian elapid venoms (23) and in this study was identified in a further six snakes. 5′-Nucleotidase activity has been demonstrated in a variety of snake venoms, including the Australian elapids (42). In support of these data, protein spots matching to mammalian 5′-nucleotidase were observed in seven different venoms including the *Oxyuranus* species. 2D gel spots corresponding to CRISPs were identified in four venoms. L-Amino-acid oxidase has been identified previously in *P. australis* venom and in this study was identified in 12 Australian elapid venoms, including *P. australis*. Protein-disulfide isomerase was identified in *P. nuchalis* (Western brown snake). Two discrete spots of ~50 kDa in the PAGE separation of the venom of *Notechis ater serventyi* (Supplemental Fig. 2C, spots 19 and 38) were matched via MS to GRP78. This protein has also been identified recently in the venom of another Australian elapid, *P. textilis* (31).

Post-translational Modification of Venom Proteins

We have shown previously that 2D PAGE separation of *P. textilis* venom proteins gives rise to multiple horizontal trains of spots for several proteins, representing differing numbers of charged groups attached post-translationally (31). This phenomenon was evident in the 2D maps for all 18 species of snake venoms in the current study. A clear example of this was observed for the heavy chain of FXa-like protease (35 kDa) present in the three *Notechis* species (Supplemental Fig. 1, A–C), the three *Pseudonaja* species (Supplemental Fig. 1, D–F), *P. porphyriacus* (Supplemental Fig. 1K), and the two *Oxyuranus* species (Supplemental Fig. 1, N and O). In *N. scutatus*, this heavy chain was observed as spots ranging in pI from 8 to 10 (Supplemental Fig. 2A, spots 26–28), whereas the predicted pI from the cDNA sequence (NCBI accession number AAX37262) is 8.7. This suggests modifications are added to the heavy chain isoforms resulting in both loss and gain of charge with only minor influence on molecular size.

[2] G. W. Birrell, S. T. H. Earl, P. P. Masci, J. de Jersey, and M. F. Lavin, manuscript in preparation.

Virtually all the proteins above 30 kDa in all 18 species were represented by multiple horizontal trains of spots.

Although a number of different post-translational modifications have been reported for snake venom proteins (33), no evidence has been presented for changes in phosphorylation status. Mammalian FVa has been shown to be phosphorylated on Ser-692, which may be important for its inactivation by activated protein C (43). This site is not present in the FVa-like molecule from *P. textilis*, but Rao *et al.* (15) have described several predicted phosphorylation sites in this molecule. In an effort to establish the phosphorylation status of venom proteins in all 18 species we used the ProQ Diamond stain, which has been reported to be specific for phosphoproteins (44). The results in Fig. 3*B* reveal the presence of strongly staining phosphorylated proteins over a wide range of molecular sizes in seven of the nine genera studied. *Acanthophis* and *Oxyuranus* displayed only low intensity staining. Curiously there was a wide range of staining intensity of protein bands within genera as clearly demonstrated by the variation among the three *Notechis* species (Fig. 3B, *lanes 1–3*). The lack of signal in the case of *N. ater niger* (Fig. 3*B*, *lane 2*) is not due to lower protein loading as the same amount of protein was used as in Fig. 1 where the silver-stained gel shows equal loading. No phosphorylation was detected for FXa-like protease heavy and light chains, which are present in abundance in *Notechis*, *Pseudonaja*, *Oxyuranus*, and other species. The identity of these phosphorylated proteins remains to be determined.

Glycoproteins were also detected using the specific stain ProQ Emerald (Fig. 3*C*). Glycosylation of a high molecular weight region corresponding in size to the FVa-like protein was particularly prominent for all three *Pseudonaja* species. Some labeling was also seen for the *Oxyuranus* species, which also contain Factor Va-like protein. As expected, neither the *Notechis*, *P. porphyriacus*, *T. carinatus*, *R. nigrescens*, nor *H. stephensii* had evidence of glycosylation in the high molecular weight region. Although all of these have prothrombinase activity and possess an FXa-like protease they do not have the FVa molecule.

The rather extensive amount of phosphorylation observed in the *Notechis* and other species such as *T. carinatus* could mean direct phosphorylation of serine, threonine, and/or tyrosine residues on the protein or the presence of phosphorylated glycan groups on glycoproteins. Accordingly we treated crude venom from these snakes with either deglycosylase (PNGase F) or phosphatase. A Coomassie-stained gel showed that deglycosylation significantly altered the pattern of protein migration (Fig. 4*A*, *lanes 2*, *5*, *8*, and *11*), whereas phosphatase treatment (*lanes 3*, *6*, *9*, and *12*) showed no discernible effect. Staining for phosphorylated proteins with ProQ Diamond revealed the same degree of loss of phosphate groups after treatment with either deglycosylase or phosphatase (Fig. 4*B*), suggesting that the phosphate groups were attached to the glycans rather than directly to the protein. The prominent band at ~35 kDa lost its ProQ Diamond reactivity for both *N. scutatus* and *N. ater serventyi* crude venoms after treatment with deglycosylase and phosphatase (Fig. 4*B*, *arrow*). We demonstrated that this band corresponds to the heavy chain of the FXa-like molecule by immunoblotting and showed that glycosidase treatment generated two discrete bands, one corresponding to the native protein (30 kDa) and a second band (27 kDa) corresponding to the calculated mass of the native protein (Fig. 4*C*). The existence of the upper 30-kDa band suggests incomplete deglycosylation. Although only two species of the FXa-like protease heavy chain were detected on the 1D gels, up to seven discrete spots corresponding to this protein were resolved by 2D PAGE. Immunoblotting with an antivenom against *N. scutatus* venom also detected these two bands in a region corresponding to the FXa-like heavy chain (Fig. 4*D*). There was also evidence of cleavage of additional proteins of higher molecular size. It is of interest that this antivenom also detected a similar distribution and cleavage pattern of proteins in the other two *Notechis* species as well as *T. carinatus* (Fig. 4*D*). The strong reactivity of the antivenom in the low molecular size region corresponding to neurotoxins is consistent with the reported ability of this antivenom to neutralize neurotoxin activity (45).

We have shown previously that fluorescently labeled lectins detect a variety of glycoprotein moieties in venom proteins from *P. textilis* (31). Using similar methodology, we studied the binding of eight different lectins to proteins separated from all 18 venoms by 1D SDS-PAGE (Fig. 5). Wheat germ agglutinin (WGA), which binds to *N*-acetylglucosamine (GlcNAc) and sialic acid, detected a protein band of 13–15 kDa corresponding to the abundant PLA_2s (Fig. 5*A*) in most species except for *T. carinatus*, *R. nigrescens*, *H. stephensii*, and one of the three *Pseudonaja* species, *Pseudonaja affinis*. This is consistent with an earlier report showing GlcNAc attached to a PLA_2 from *O. scutellatus* (46). There was very prominent staining of a protein of lower molecular size corresponding to the Kunitz-like serine protease inhibitors in three of the 18 species, two of the three *Pseudonaja* (again not evident in *P. affinis*) and *A. antarcticus*. A similar pattern of staining in this molecular size region was also evident in the same species using ConA (mannose and glucose), SNA (sialic acid), *Dolichos biflorus* agglutinin (*N*-acetylgalactosamine), UEA (fucose), soybean agglutinin (*N*-acetylgalactosamine and galactose), RCA120 (*N*-acetylgalactosamine and galactose), and peanut agglutinin (galactose) binding. However in the case of ConA binding, more prominent labeling was observed for *T. carinatus*, *R. nigrescens*, and *H. stephensii.* A prominent binding band at ~22 kDa was detected by SNA and UEA (Fig. 5, *C* and *E*) that detect sialic acid or one of its constituents. Although this band is in a region corresponding to the light chain of the FXa-like protease, it is unlikely that this protein has been modified because it was detected in several species that do not have a prothrombin activator. Lectin binding in the high molecular weight region was prominent for WGA and ConA for the vast majority of the 18 species, whereas very little reactivity was

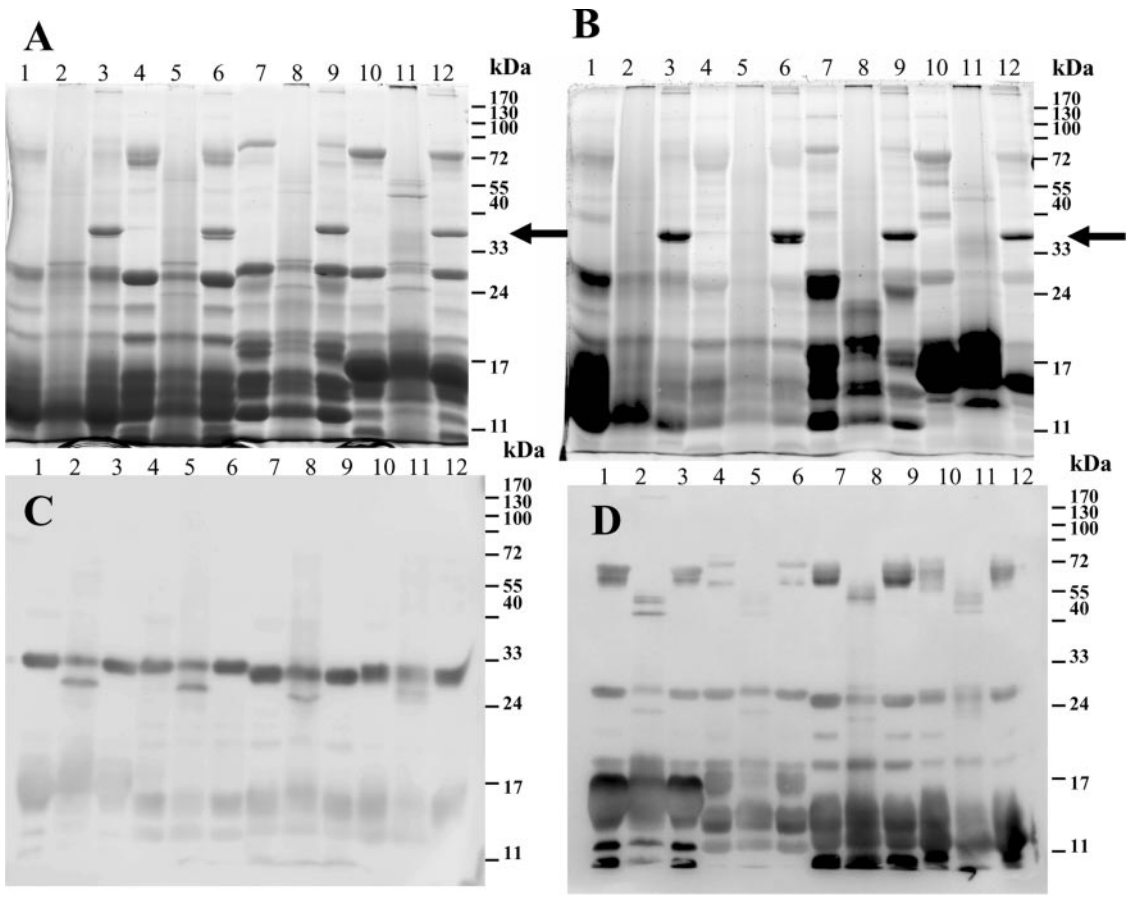

FIG. 4. **Effect of deglycosylation and dephosphorylation.** Venom samples from four species were treated with glycosidase and phosphatase and separated by 12% SDS-PAGE. *A*, Coomassie-stained gel. *Lane 1*, *N. scutatus* venom; *lane 2*, deglycosylated *N. scutatus* venom; *lane 3*, dephosphorylated *N. scutatus* venom; *lane 4*, *N. ater niger* venom; *lane 5*, deglycosylated *N. ater niger* venom; *lane 6*, dephosphorylated *N. ater niger* venom; *lane 7*, *N. ater serventyi* venom; *lane 8*, deglycosylated *N. ater serventyi* venom; *lane 9*, dephosphorylated *N. ater serventyi* venom; *lane 10*, *T. carinatus* venom; *lane 11*, deglycosylated *T. carinatus* venom; *lane 12*, dephosphorylated *T. carinatus* venom. *B*, samples from *A* run on a parallel gel and stained with ProQ Diamond phosphoprotein-specific stain. *C*, samples from *A* run on a parallel gel and immunoblotted with antiserum to *P. textilis* Factor Xa-like heavy chain. *D*, samples from *A* run on a parallel gel and immunoblotted with antivenom to *N. scutatus*. The band marked with an *arrow* in *A* and *B*, *lanes 3*, *6*, *9*, and *12*, is the Antarctic phosphatase.

seen in this region for the other six lectins. However, there was some reactivity seen with RCA120 (*N*-acetylgalactosamine and galactose) in a few species.

DISCUSSION

This study represents the first thorough proteomics study of the venoms from the major species of Australian elapid snakes. In all, we identified members of 17 different protein families in nine different genera. The relative lack of complexity allowed us via MS to identify the majority of the proteins separated by 2D PAGE. Proteins were isolated with isoelectric points from 3 to 10 and with molecular sizes ranging from 5 up to 160 kDa. It was evident that there were conserved patterns of protein staining within genera. The most obvious example of this was for the prothrombin activator, which is present in *Notechis*, *Pseudonaja*, *Oxyuranus*, only one of the *Pseudechis* species, *T. carinatus*, *R. nigrescens*, and *H. stephensii*. Coagulopathy is thought to represent the major cause of morbidity in individuals envenomated by these snakes (8). In the case of *Pseudonaja*, all three species had bands that corresponded to components of the prothrombin activator: FVa-like protein (at 160 kDa), the heavy chain of FXa-like protein (at 35 kDa), and the light chain of the FXa-like protein (at 24 kDa). This was verified by both mass spectrometry and use of specific antisera. These proteins were also evident in the two *Oxyuranus* species but were present in lesser amount than in the *Pseudonaja* species as observed previously (47). St Pierre *et al.* (47) used comparative analysis of cDNA clones to reveal a three-way identity of 91% at the amino acid level of FXa-like protein between the two *Oxyuranus* species and *P. textilis*. In *P. textilis*, the prothrombin activator (complex of FXa-like and FVa-like) represents up to 30% of the dry weight of the venom (6). Although the *Notechis* species also contain procoagulant activity, the FVa-like, non-enzymatic component of the activator is absent. However, the amount of the FXa-like protease in these species was similar to that observed in *Pseudonaja*.

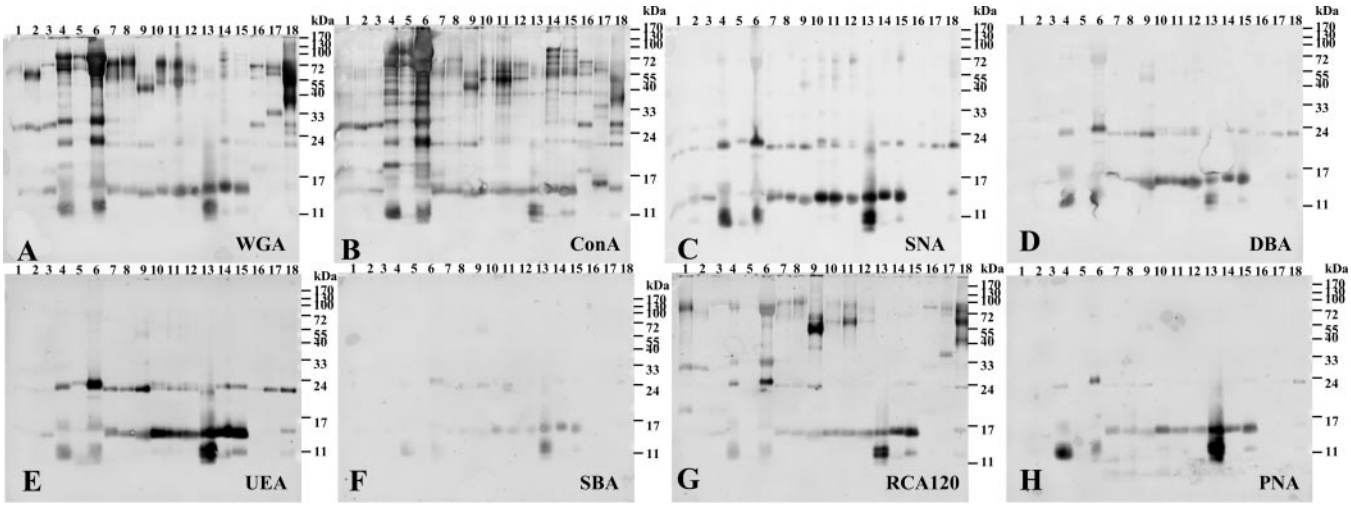

FIG. 5. **Lectin binding.** Venom proteins were separated by 2D PAGE, and proteins were transferred to nitrocellulose membrane to examine binding of various lectins. *Lane 1*, *N. scutatus*; *lane 2*, *N. ater niger*; *lane 3*, *N. ater serventyi*; *lane 4*, *P. nuchalis*; *lane 5*, *P. affinis*; *lane 6*, *P. inframacula*; *lane 7*, *A. superbus*; *lane 8*, *A. ramsayi*; *lane 9*, *P. porphyriacus*; *lane 10*, *P. australis*; *lane 11*, *P. guttatus*; *lane 12*, *P. colletti*; *lane 13*, *A. antarcticus*; *lane 14*, *O. scutellatus*; *lane 15*, *O. microlepidotus*; *lane 16*, *T. carinatus*; *lane 17*, *R. nigrescens*; *lane 18*, *H. stephensii*. *A*, WGA (sialic acid and GlcNAc); *B*, ConA (mannose and glucose); *C*, SNA (α2–3 sialic acid); *D*, *D. biflorus* agglutinin (*DBA*) (GalNAc); *E*, UEA (fucose); *F*, soybean agglutinin (*SBA*) (GalNAc and galactose); *G*, RCA120 (GalNAc and galactose); *H*, peanut agglutinin (*PNA*) (galactose) (Lectin binding specificities are shown in parentheses.).

The size difference between this protein band between the two genera can be explained by the presence of an additional 14 amino acids in the *Pseudonaja* species (47).

Using mass spectrometry, we identified multiple isoforms of PLA_2s in all of the Australian elapid venoms. Snake venom PLA_2s have been implicated in a number of physiological processes including hemorrhage, myotoxicity, hemolytic and hypotensive activities as well as edema, platelet aggregation, cardiotoxicity, and pre- and postsynaptic neurotoxicity (48). These proteins have been identified in almost all snake venoms, regardless of genera, and it is the PLA_2 enzymes of the Australian elapids that constitute the single most widely studied family of toxins from these snakes (49). Consequently protein sequence data for these enzymes is readily accessible for PMM matching. Multiple protein spots corresponding to these enzymes were detected across the entire pI range of 3–10 and generally in the molecular size region of 13–15 kDa. Previous evidence from Australian elapids, including *P. australis*, *N. scutatus*, and *A. superbus*, indicates that there are multiple PLA_2 isoforms within the venom of these snakes (50, 51). A more recent study using cDNA cloning in *O. scutellatus* identified eight clones coding for PLA_2 sequence variants with predicted mature protein sizes ranging from 13.3 to 14.4 kDa and ranging in pI values from 4.6 to 8.4 (34), whereas in the present study, PLA_2s ranged in size from 13 to 50 kDa and in pI values from 3 to 10.

The PMM and MS/MS data obtained here (Supplemental Table 1) represents a large number of individual peptides that correspond to a variety of individual PLA_2s, consistent with previous data that provide evidence for multiple variants of these enzymes (46). A number of larger molecular size spots from 2D PAGE also contained PLA_2 peptides. However this may be due to incomplete dissociation of molecular complexes during the 2D PAGE procedure, the existence of novel higher molecular size forms, and/or post-translational modifications such as glycosylation as suggested by the lectin binding experiments (Fig. 5). An example of this is the Taipoxin complex, a potently active toxin complex within the venom of *O. scutellatus* composed of three PLA_2 chains: α, β, and a glycosylated γ-chain (46). The γ-chain has a predicted molecular size of 14.6 kDa (Swiss-Prot accession number P00616) yet it migrates to ~25 kDa on 2D PAGE (Supplemental Fig. 2, spots 1 and 2) likely due to the existence of *N*-linked carbohydrate structure (46). The γ-chain has been observed previously to migrate at 26.9 kDa and has a predicted pI of 4.1, which is consistent with that observed on the 2D PAGE (Supplemental Fig. 2N, spots 1 and 2). Natriuretic peptides were the only known protein family not found in the present study possibly due to their small molecular size (35–39 amino acids) (25).

Novel Snake Venom Proteins—In this study we identified two novel proteins, protein-disulfide isomerase (PDI) and a PLA_2 inhibitor not previously reported in the venom of any snake. PDIs are multidomain, multifunctional members of the thioredoxin superfamily that catalyze thiol oxidation, disulfide reduction, and isomerization (52). Isomerization occurs directly by intramolecular disulfide rearrangement or through cycles of reduction and oxidation (53). Twelve peptides corresponding to a PDI sequence in the NCBI protein database were identified in a protein spot from *P. nuchalis* whose molecular size corresponds with the predicted size of the mature

protein (58 kDa). The database sequence represents a conceptual translation of a cDNA sequence isolated from the venom gland of *O. scutellatus* (NCBI accession number AAY33972) and has not yet been published.[3] Because several of the toxic components of the venom are known to contain essential disulfide bridges (*e.g.* PLA_2s, textilinins, and prothrombin activator components), it is possible that PDI plays a role in the synthesis and maintenance of these proteins in their active structures in the venom gland. Interestingly human protein-disulfide isomerase has been shown recently to suppress tissue factor coagulant activity by targeting a disulfide bond in tissue factor that is critical for its coagulation activity (54). This suggests that protein-disulfide isomerase could also play a role as an anticoagulant in the venom.

The second protein, PLA_2 inhibitor, has not been described in snake venom, but there are several reports of the existence of such an inhibitor in the blood of American, Asian, and Australian snakes (35, 55). Hains and Broady (35) purified and characterized PLA_2 inhibitors from the sera of *N. scutatus*, *N. ater niger*, *N. ater serventyi*, *O. scutellatus*, *O. microlepidotus*, and *P. textilis*. They proposed that PLA_2 inhibitors exist in snake blood as a defense mechanism against the toxic PLA_2 enzymes produced in the venom glands. We were therefore surprised to find the protein in the venom of *P. nuchalis*. Three spots were identified corresponding to two forms of the α-chain and one of the β-chain suggesting that it is closely related, if not identical, to the serum inhibitor. It is not immediately clear why a PLA_2 inhibitor would be present in the venom particularly in such low abundance compared with PLA_2s themselves. However, because not all snake venom PLA_2 enzymes have PLA_2 activity, it is possible that the inhibitor is specific for only a subclass that could damage the epithelial lining of the venom gland where they are produced. It would seem unlikely that the PLA_2 inhibitor that we identified was the result of serum contamination of the venom sample as no other serum proteins were detected in this or any other venom sample.

Novel Proteins in Australian Snake Venoms—Four families of proteins (vespryn, metalloproteinase, C-type lectin, and acetylcholinesterase) that have been detected previously in snake venoms from other regions are reported here for the first time in venoms of Australian elapids. Pung *et al.* (38) first described Ohanin as a novel protein from *O. hannah* (king cobra) venom and subsequently named this protein subfamily "vespryns" (56). They cloned a full-length cDNA for this protein of 1558 bp. The predicted protein contained a propeptide of 22 amino acids at the N terminus and an additional "propeptide" of 63 amino acids at the C terminus. The mature protein was 107 amino acids in length. It was not clear from the report of Pung *et al.* (56) how the C-terminal region was identified as a propeptide. Ohanins are related in sequence to PRY-SPRY (B30.2-like) domain-containing proteins that include mammalian ryanodine receptor subtypes and the dual specificity kinase, splA, found in *Dictyostelium discoideum.* There are only three protein sequences in the public domain with >50% shared identity to Ohanin. These related proteins are Thaicobrin from *Naja kaouthia* (UniProt accession number P82885), an Ohanin-like protein from *Lachesis muta* (GenBank™ accession number DQ396476), and vespryn-POGU1 from the Australian bearded dragon, *Pogona barbata* (GenBank accession number AAZ75637) (57). Indeed Fry *et al.* (57) used cDNA libraries and phylogenetic analysis of transcripts such as vespryn-POGU1 to show that nine toxin types are shared between lizards and snakes, supporting their theory for a single, early origin of the venom system in lizards and snakes.

SVMPs have been identified in some viperid and elapid venoms and are reported to play key roles in the development of such symptoms as hemorrhage, edema, hypotension, hypovolemia, inflammation, and necrosis (39). SVMPs were found in seven of the 18 species examined here. The molecular size varied from 60 to 80 kDa with pI in the range of 4–6. The mass spectrometry data matched to snake venom metalloproteinases from the M12B reprolysin subfamily. This subfamily possesses three different domains: the reprolysin catalytic domain, a disintegrin domain that inhibits the binding of ligands to integrin receptors, and a cysteine-rich domain. The peptide sequences identified here were found to match these regions. It is likely that these enzymes are present in most Australian elapid snakes because it was possible to successfully amplify metalloproteinase cDNAs using PCR primers designed from the cobra cDNA sequence (GenBank accession number AY101383)[4] from seven different genera.[2] The presence of these enzymes in a wide variety of snakes suggests that they play important roles in toxicity. SVMPs possess multidomain functions, and it has been shown that the catalytic site is not the only crucial domain enabling this class of toxin to cause hemorrhage and inflammation (58). This is evidenced by a form of SVMP, jararhagin C, that lacks the catalytic domain yet is capable of activating an acute inflammatory response in a mouse model (58). Platelets are the main target of these enzymes in the disruption of hemostasis that occurs by inhibition of interaction with collagen and/or von Willebrand factor using various mechanisms that result in systemic bleeding.

C-type lectins were also identified in Australian elapid snake venoms for the first time. These calcium-dependent sugar-binding proteins have been characterized extensively from Viperidae venoms. However, only a limited number of C-lectins have been identified in Elapidae venoms, and these remain to be fully characterized. Viper venom C-lectins have been shown to cause edema and increased vascular permeability and to agglutinate erythrocytes (59, 60). The C-lectins identified from the Australian elapids match most closely to a

[3] R. E. Welton and J. N. Burnell, unpublished data.

[4] D. Sako and G. D. Shaw, unpublished data.

galactose-binding C-lectin sequence from the krait *B. fasciatus*, suggesting that they also bind this sugar. Although this protein was only identified in six of the 18 species, C-lectin cDNAs have been successfully amplified from seven different genera of Australian elapids[5] suggesting that this protein is a common venom component in these snakes.

Acetylcholinesterase activity has been reported in several snake venoms previously; however, only one snake venom acetylcholinesterase has been isolated and characterized, that from *B. fasciatus* (41). Acetylcholinesterase was identified via MS in five species as an ~80-kDa train of spots. This molecular size is consistent with that of *B. fasciatus* acetylcholinesterase, which is known to contain *N*-linked glycosylation. Glycosylation of the Australian forms may account for the train of spots observed in this study.

Post-translational Modifications—We have provided evidence here for at least three types of post-translational modification in the venom proteins from the different genera of the Australian elapids: phosphorylation, glycosylation, and γ-carboxylation. Only one previous report, from our laboratory, has provided evidence for the presence of phosphorylated snake venom proteins (31). It seems unlikely that this was artifactual binding because the phosphorylated spots correlated well with protein staining, and the ProQ Diamond technique has been shown to be very specific for protein phosphorylation (44). This method of staining failed to detect phosphate in DNA or RNA and did not detect sulfated glycans. Birrell *et al.* (31) demonstrated that ProQ Diamond detected proteins in *P. textilis* that corresponded in size to the major toxins in that venom including FVa-like and FXa-like proteins as well as PLA_2s. In this study, phosphorylated proteins were detected in most snake species, and they varied in molecular size from ~15 kDa up to greater than 100 kDa. Proteins are normally phosphorylated as parts of signaling mechanisms, leading to alterations in their conformation and/or their capacity to interact with other proteins. In this case it is more likely that the phosphorylated species detected are glycoproteins containing phosphorylated carbohydrate. Phosphorylated glycoproteins are widely distributed in nature and play key roles in a variety of biological processes. For example, soluble lysosomal proteins are synthesized in the endoplasmic reticulum and are cotranslationally glycosylated on specific asparagine residues. As these proteins move through the secretory pathway, they are selectively recognized by a phosphotransferase that initiates a two-step reaction that results in the generation of the mannose 6-phosphate modification on specific *N*-linked oligosaccharides. This modification causes the lysosomal protein to bind mannose 6-phosphate receptors as part of the process of endocytosis and lysosomal targeting (61). Another example of a glycoprotein in which the carbohydrate moiety is phosphorylated is cathepsin D (62). Cathepsin D is an aspartic peptidase that is found at elevated levels in some tumor tissues; however, the role of the phosphorylated carbohydrate is unknown. To understand the role of putative phosphorylated glycoproteins in snake venom it will be necessary to identify these proteins, determine whether phosphorylation status influences their interaction with other proteins, and determine whether phosphorylation alters the function of these proteins in specific functional assays, *e.g.* proteolytic activity, coagulability, or neurotoxicity.

Glycosylation of proteins was detected using lectin binding and ProQ Emerald staining. A high molecular weight signal was detected in the venom from several species that corresponded to FVa-like protein in 1D SDS-PAGE (Fig. 3*C*). This is in good agreement with previous results using 2D PAGE with *P. textilis* that detected spots of the same molecular size as FVa-like and FXa-like proteins. This was confirmed with ConA (glucose- and mannose-specific) and WGA (GlcNAc- and sialic acid-specific) lectins. It is not evident what role this modification may have on components of the prothrombin activator, but it is possible that the abundance of glycosylation of these proteins influences their stability and/or function. Increased stability would result in greater diffusion and transport of glycosylated toxins upon envenomation.

Clinical Implications—In general, the results of this study support previous clinical data from patients envenomated by Australian elapid species. The major clinical effects of Australian elapid venoms include paralysis, coagulopathy, and muscle destruction, and these symptoms can be attributed largely to three groups of proteins, neurotoxins, prothrombin activators, and PLA_2s (8). Inspection of the 2D maps showed that these three groups of proteins account for a large proportion of the total venom proteins, confirming their high abundance. We identified several venom proteins not known previously in Australian snake venoms, but reported in other snake venoms, that may have clinical manifestations. For example, SVMPs from several Viperidae venoms can cause hemorrhage, edema, hypotension, hypovolemia, inflammation, and necrosis (39). Similarly C-type lectins that have also been characterized from Viperidae venoms can cause edema and increased vascular permeability and can agglutinate erythrocytes (59, 60). Thus it may be possible that some clinical effects attributed previously to PLA_2s, such as necrosis and inflammation, are in fact caused by the SVMPs. The clinical effects of other proteins identified in Australian elapid snakes for the first time here also remain to be determined. Moreover some of these proteins may have unique activities applicable to the development of new human therapeutics.

Conclusions—We have described here a comprehensive analysis of the venom proteins of 18 Australian elapid snakes that are among the most venomous snakes worldwide. This represents a significant advance in the proteomics of snake venom proteins and an important resource for comparison of protein patterns both between species of the same genera and across genera. The vast majority of these proteins were

[5] S. T. H. Earl, G. W. Birrell, P. P. Masci, J. de Jersey, and M. F. Lavin, manuscript in preparation.

identified using mass spectrometry of spots isolated from 2D PAGE.

Because a significant number of the peptides isolated failed to match with any peptides in the NCBI non-redundant protein database it was necessary to use *de novo* peptide sequencing by tandem mass spectrometry, which significantly increased the total number of proteins identified. In all, we identified six previously undescribed families of proteins in Australian elapid snakes. Some of these, including vespryns, metalloproteinases, C-type lectins, and 5′-nucleotidase were described previously in non-Australian snake venoms (38, 63–66). Protein-disulfide isomerase and PLA_2 inhibitor have not been reported previously in any snake venom, although a very recent report has described a cDNA coding for PDI (67), and a PLA_2 inhibitor has been reported in snake serum (36). In addition to identifying all the major venom proteins in the Australian snakes, we partially characterized them using specific antibodies, phosphoprotein- and glycoprotein-specific stains, enzymatic digestion, lectin binding, and antivenom reactivity. Further characterization of these proteins will assist in delineating their role in the venoms, their clinical effects, and their potential as therapeutic agents.

Acknowledgments—We acknowledge Tracey Laing and Danielle Gunn for secretarial assistance.

* This work was supported by QRxPharma and the Australian Research Council Linkage Program. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[S] The on-line version of this article (available at http://www.mcponline.org) contains supplemental material.

¶ Both authors contributed equally to this work.

** Present address: The Queensland Inst. of Medical Research, P. O. Royal Brisbane Hospital, Brisbane 4029, Australia.

¶¶ To whom correspondence should be addressed: The Queensland Cancer Fund Research Unit, The Queensland Inst. of Medical Research, P. O. Box Royal Brisbane Hospital, Herston, Brisbane 4029, Australia. Tel.: 617-3362-0341; Fax: 617-3362-0106; E-mail: martin.lavin@qimr.edu.au.

REFERENCES

1. Underwood, G. (1979) in *Handbook of Experimental Pharmacology* (Lee, Y., ed) pp. 15–40, Springer, Berlin
2. Cushman, D. W., and Ondetti, M. A. (1999) Design of angiotensin converting enzyme inhibitors. *Nat. Med.* **5,** 1110–1112
3. O'Shea, J. C., and Tcheng, J. E. (2002) Eptifibatide: a potent inhibitor of the platelet receptor integrin glycoprotein IIb/IIIa. *Expert Opin. Pharmacother.* **3,** 1199–1210
4. Burger, K. M., and Tuhrim, S. (2004) Antithrombotic trials in acute ischaemic stroke: a selective review. *Expert Opin. Emerg. Drugs* **9,** 303–312
5. Toombs, C. F. (2001) Alfimeprase: pharmacology of a novel fibrinolytic metalloproteinase for thrombolysis. *Haemostasis* **31,** 141–147
6. Masci, P. P., Whitaker, A. N., and de Jersey, J. (1988) Purification and characterization of a prothrombin activator from the venom of the Australian brown snake, *Pseudonaja textilis textilis*. *Biochem. Int.* **17,** 825–835
7. Filippovich, I., Sorokina, N., Masci, P. P., de Jersey, J., Whitaker, A. N., Winzor, D. J., Gaffney, P. J., and Lavin, M. F. (2002) A family of textilinin genes, two of which encode proteins with antihaemorrhagic properties. *Br. J. Haematol*. **119,** 376–384
8. Sutherland, S. K., and Tibballs, J. (2001) *Australian Animal Toxins*, pp. 59–219, Oxford University Press, Melbourne, Australia
9. Russell, F. E. (1980) Snake venom poisoning in the United States. *Annu. Rev. Med*. **31,** 247–259
10. Flight, S., Mirtschin, P., and Masci, P. P. (2006) Comparison of active venom components between eastern brown snakes collected from South Australia and Queensland. *Ecotoxicology* **15,** 133–141
11. Kini, R. M., Morita, T., and Rosing, J. (2001) Classification and nomenclature of prothrombin activators isolated from snake venoms—On behalf of the Registry of Exogenous Hemostatic Factors of the Scientific and Standardization Committee of the International Society on Thrombosis and Haemostasis. *Thromb. Haemostasis* **86,** 710–711
12. Kini, R. M. (2005) The intriguing world of prothrombin activators from snake venom. *Toxicon* **45,** 1133–1145
13. Joseph, J. S., and Kini, R. M. (2001) Snake venom prothrombin activators homologous to blood coagulation factor Xa. *Haemostasis* **31,** 234–240
14. Rao, V. S., and Kini, R. M. (2002) Pseutarin C, a prothrombin activator from *Pseudonaja textilis* venom: its structural and functional similarity to mammalian coagulation factor Xa-Va complex. *Thromb. Haemostasis* **88,** 611–619
15. Rao, V. S., Swarup, S., and Kini, R. M. (2003) The nonenzymatic subunit of pseutarin C, a prothrombin activator from eastern brown snake (*Pseudonaja textilis*) venom, shows structural similarity to mammalian coagulation factor V. *Blood* **102,** 1347–1354
16. Masci, P. P., Whitaker, A. N., Sparrow, L. G., de Jersey, J., Winzor, D. J., Watters, D. J., Lavin, M. F., and Gaffney, P. J. (2000) Textilinins from *Pseudonaja textilis textilis*. Characterization of two plasmin inhibitors that reduce bleeding in an animal model. *Blood Coagul. Fibrinolysis* **11,** 385–393
17. Fry, B. G. (1999) Structure-function properties of venom components from Australian elapids. *Toxicon* **37,** 11–32
18. Southcott, R. N., and Coulter, A. R. (1979) in *Neurotoxins, Fundamental and Clinical Advances* (Chubb, I. W., and Geffen, L. B., eds) p. 272, Adelaide University Union Press, Adelaide, Australia
19. Tyler, M. I., Retson Yip, K. V., Gibson, M. K., Barnett, D., Howe, E., Stocklin, R., Turnbull, R. K., Kuchel, T., and Mirtschin, P. (1997) Isolation and amino acid sequence of a new long-chain neurotoxin with two chromatographic isoforms (Aa e1 and Ae e2) from the venom of the Australian death adder (*Acanthophis antarcticus*). *Toxicon* **35,** 555–562
20. Brown, R. L., Haley, T. L., West, K. A., and Crabb, J. W. (1999) Pseudechetoxin: a peptide blocker of cyclic nucleotide-gated ion channels. *Proc. Natl. Acad. Sci. U. S. A.* **96,** 754–759
21. Yamazaki, Y., Brown, R. L., and Morita, T. (2002) Purification and cloning of toxins from elapid venoms that target cyclic nucleotide-gated ion channels. *Biochemistry* **41,** 11331–11337
22. Stiles, B. G., Sexton, F. W., and Weinstein, S. A. (1991) Antibacterial effects of different snake venoms: purification and characterization of antibacterial proteins from Pseudechis australis (Australian king brown or mulga snake) venom. *Toxicon* **29,** 1129–1141
23. Earl, S. T., Birrell, G. W., Wallis, T. P., St Pierre, L. D., Masci, P. P., de Jersey, J., Gorman, J. J., and Lavin, M. F. (2006) Post-translational modification accounts for the presence of varied forms of nerve growth factor in Australian elapid snake venoms. *Proteomics* **6,** 6554–6565
24. Fry, B. G., Wickramaratana, J. C., Lemme, S., Beuve, A., Garbers, D., Hodgson, W. C., and Alewood, P. (2005) Novel natriuretic peptides from the venom of the Inland Taipan (*Oxyuranus microlepidotus*): isolation, chemical and biological characterisation. *Biochem. Biophys. Res. Commun.* **327,** 1011–1015
25. St Pierre, L., Flight, S., Masci, P. P., Hanchard, K. J., Lewis, R. J., Alewood, P. F., de Jersey, J., and Lavin, M. F. (2006) Cloning and characterisation of natriuretic peptides from the venom glands of Australian elapids. *Biochimie* (*Paris*) **88,** 1923–1931
26. Filippovich, I., Sorokina, N., St Pierre, L., Flight, S., de Jersey, J., Perry, N., Masci, P. P., and Lavin, M. F. (2005) Cloning and functional expression of venom prothrombin activator protease from Pseudonaja textilis with whole blood procoagulant activity. *Br. J. Haematol*. **131,** 237–246
27. Shevchenko, A., Wilm, M., Vorm, O., and Mann, M. (1996) Mass spectrometric sequencing of proteins silver-stained polyacrylamide gels. *Anal. Chem*. **68,** 850–858
28. Gharahdaghi, F., Weinberg, C. R., Meagher, D. A., Imai, B. S., and Mische, S. M. (1999) Mass spectrometric identification of proteins from silver-

stained polyacrylamide gel: a method for the removal of silver ions to enhance sensitivity. *Electrophoresis* **20,** 601–605
29. Ma, B., Zhang, K., Hendrie, C., Liang, C., Li, M., Doherty-Kirby, A., and Lajoie, G. (2003) PEAKS: powerful software for peptide de novo sequencing by tandem mass spectrometry. *Rapid Commun. Mass Spectrom*. **17,** 2337–2342
30. Nawarak, J., Phutrakul, S., and Chen, S. T. (2004) Analysis of lectin-bound glycoproteins in snake venom from the Elapidae and Viperidae families. *J. Proteome Res.* **3,** 383–392
31. Birrell, G. W., Earl, S., Masci, P. P., de Jersey, J., Wallis, T. P., Gorman, J. J., and Lavin, M. F. (2006) Molecular diversity in venom from the Australian brown snake, Pseudonaja textilis. *Mol. Cell. Proteomics* **5,** 379–389
32. Serrano, S. M. T., Shannon, J. D., Wang, D. Y., Camargo, A. C. M., and Fox, J. W. (2005) A multifaceted analysis of viperid snake venoms by two-dimensional gel electrophoresis: an approach to understanding venom proteomics. *Proteomics* **5,** 501–510
33. Li, S., Wang, J., Zhang, X., Ren, Y., Wang, N., Zhao, K., Chen, X., Zhao, C., Li, X., Shao, J., Yin, J., West, M. B., Xu, N., and Liu, S. (2004) Proteomic characterization of two snake venoms: Naja naja atra and Agkistrodon halys. *Biochem. J.* **384,** 119–127
34. St Pierre, L., Woods, R., Earl, S., Masci, P. P., and Lavin, M. F. (2005) Identification and analysis of venom gland-specific genes from the coastal taipan (*Oxyuranus scutellatus*) and related species. *Cell. Mol. Life Sci.* **62,** 2679–2693
35. Hains, P. G., and Broady, K. W. (2000) Purification and inhibitory profile of phospholipase A2 inhibitors from Australian elapid sera. *Biochem. J.* **346,** 139–146
36. Dunn, R. D., and Broady, K. W. (2001) Snake inhibitors of phospholipase A_2 enzymes. *Biochim. Biophys. Acta* **1533,** 29–37
37. Rao, V. S., Joseph, J. S., and Kini, R. M. (2003) Group D prothrombin activators from snake venom are structural homologues of mammalian blood coagulation factor Xa. *Biochem. J.* **369,** 635–642
38. Pung, Y. F., Wong, P. T., Kumar, P. P., Hodgson, W. C., and Kini, R. M. (2005) Ohanin, a novel protein from king cobra venom, induces hypolocomotion and hyperalgesia in mice. *J. Biol. Chem.* **280,** 13137–13147
39. Fox, J. W., and Serrano, S. M. (2005) Structural considerations of the snake venom metalloproteinases, key members of the M12 reprolysin family of metalloproteinases. *Toxicon* **45,** 969–985
40. Swenson, S., and Markland, F. S., Jr. (2005) Snake venom fibrin(ogen)olytic enzymes. *Toxicon* **45,** 1021–1039
41. Cousin, X., Bon, S., Duval, N., Massoulie, J., and Bon, C. (1996) Cloning and expression of acetylcholinesterase from *Bungarus fasciatus* venom. New type of COOH-terminal domain involvement of a positively charged residue in the peripheral site. *J. Biol. Chem.* **271,** 15099–15108
42. Tan, N. H., and Ponnudurai, G. (1990) A comparative study of the biological properties of Australian elapid venoms. *Comp. Biochem. Physiol. C Comp. Pharmacol. Toxicol.* **97,** 99–106
43. Kalafatis, M. (1998) Identification and partial characterization of factor Va heavy chain kinase from human platelets. *J. Biol. Chem.* **273,** 8459–8466
44. Steinberg, T. H., Agnew, B. J., Gee, K. R., Leung, W. Y., Goodman, T., Schulenberg, B., Hendrickson, J., Beechem, J. M., Haugland, R. P., and Patton, W. F. (2003) Global quantitative phosphoprotein analysis using multiplexed proteomics technology. *Proteomics* **3,** 1128–1144
45. Ramasamy, S., Fry, B. G., and Hodgson, W. C. (2005) Neurotoxic effects of venoms from seven species of Australasian black snakes (Pseudechis): efficacy of black and tiger snake antivenoms. *Clin. Exp. Pharmacol. Physiol*. **32,** 7–12
46. Fohlman, J., Eaker, D., Karlsson, E., and Thesleff, S. (1976) Taipoxin, an extremely potent presynaptic neurotoxin from venom of Australian snake taipan (*Oxyuranus scutellatus*)—isolation, characterization, quaternary structure and pharmacological properties. *Eur. J. Biochem.* **68,** 457–469
47. St Pierre, L., Masci, P. P., Filippovich, I., Sorokina, N., Marsh, N., Miller, D. J., and Lavin, M. F. (2005) Comparative analysis of prothrombin activators from the venom of Australian elapids. *Mol. Biol. Evol.* **22,** 1853–1864
48. Arni, R. K., and Ward, R. J. (1996) Phospholipase A2—a structural review. *Toxicon* **34,** 827–841
49. Harris, J. B. (1997) in *Venomous Snakes, Ecology, Evolution and Snakebite* (Thorpe, R. S., Wuster, W., and Malhotra, A., eds) pp. 235–250, Clarendon Press, Oxford, UK
50. Francis, B., Coffield, J. A., Simpson, L. L., and Kaiser, I. I. (1995) Amino acid sequence of a new type of toxic phospholipase A2 from the venom of the Australian tiger snake (Notechis scutatus scutatus). *Arch. Biochem. Biophys*. **318,** 481–488
51. Takasaki, C., Suzuki, J., and Tamiya, N. (1990) Purification and properties of several phospholipases A_2 from the venom of Australian king brown snake (*Pseudechis australis*). *Toxicon* **28,** 319–327
52. Freedman, R. B., Klappa, P., and Ruddock, L. W. (2002) Protein disulfide isomerases exploit synergy between catalytic and specific binding domains. *EMBO Rep*. **3,** 136–140
53. Schwaller, M., Wilkinson, B., and Gilbert, H. F. (2003) Reduction-reoxidation cycles contribute to catalysis of disulfide isomerization by protein-disulfide isomerase. *J. Biol. Chem.* **278,** 7154–7159
54. Ahamed, J., Versteeg, H. H., Kerver, M., Chen, V. M., Mueller, B. M., Hogg, P. J., and Ruf, W. (2006) Disulfide isomerization switches tissue factor from coagulation to cell signaling. *Proc. Natl. Acad. Sci. U. S. A.* **103,** 13932–13937
55. Fortes-Dias, C. L., Barcellos, C. J., and Estevao-Costa, M. I. (2003) Molecular cloning of a γ-phospholipase A2 inhibitor from Lachesis muta muta (the bushmaster snake). *Toxicon* **41,** 909–917
56. Pung, Y. F., Kumar, S. V., Rajagopalan, N., Fry, B. G., Kumar, P. P., and Kini, R. M. (2006) Ohanin, a novel protein from king cobra venom: its cDNA and genomic organization. *Gene* (*Amst.*) **371,** 246–256
57. Fry, B. G., Vidal, N., Norman, J. A., Vonk, F. J., Scheib, H., Ramjan, S. F., Kuruppu, S., Fung, K., Hedges, S. B., Richardson, M. K., Hodgson, W. C., Ignjatovic, V., Summerhayes, R., and Kochva, E. (2006) Early evolution of the venom system in lizards and snakes. *Nature* **439,** 584–588
58. Clissa, P. B., Lopes-Ferreira, M., Della-Casa, M. S., Farsky, S. H., and Moura-da-Silva, A. M. (2006) Importance of jararhagin disintegrin-like and cysteine-rich domains in the early events of local inflammatory response. *Toxicon* **47,** 591–596
59. Komori, Y., Nikai, T., Tohkai, T., and Sugihara, H. (1999) Primary structure and biological activity of snake venom lectin (APL) from Agkistrodon p. piscivorus (eastern cottonmouth). *Toxicon* **37,** 1053–1064
60. Panunto, P. C., da Silva, M. A., Linardi, A., Buzin, M. P., Melo, S. E., Mello, S. M., Prado-Franceschi, J., and Hyslop, S. (2006) Biological activities of a lectin from Bothrops jararacussu snake venom. *Toxicon* **47,** 21–31
61. Sleat, D. E., Wang, Y., Sohar, I., Lackland, H., Li, Y., Li, H., Zheng, H., and Lobel, P. (2006) Identification and validation of mannose 6-phosphate glycoproteins in human plasma reveals a wide range of lysosomal and non-lysosomal proteins. *Mol. Cell. Proteomics* **5,** 1942–1956
62. Ohhira, M., Gasa, S., Makita, A., Sekiya, C., and Namiki, M. (1991) Elevated carbohydrate phosphotransferase activity in human hepatoma and phosphorylation of cathepsin D. *Br. J. Cancer* **63,** 905–908
63. Bjarnason, J. B., and Fox, J. W. (1995) Snake venom metalloendopeptidases: reprolysins. *Methods Enzymol.* **248,** 345–368
64. Hogue-Angeletti, R. A., Frazier, W. A., Jacobs, J. W., Niall, H. D., and Bradshaw, R. A. (1976) Purification, characterization, and partial amino acid sequence of nerve growth factor from cobra venom. *Biochemistry* **15,** 26–34
65. Clemetson, K. J., Lu, Q., and Clemetson, J. M. (2005) Snake C-type lectin-like proteins and platelet receptors. *Pathophysiol. Haemost. Thromb*. **34,** 150–155
66. Nikolskaia, I. I., Kislina, O. S., and Tikhonenko, T. I. (1964) Separation of 5′-nucleotidase from interfering enzymes in snake venom. *Dokl. Akad. Nauk SSSR* **157,** 475–477
67. Zhang, B., Liu, Q., Yin, W., Zhang, X., Huang, Y., Luo, Y., Qiu, P., Su, X., Yu, J., Hu, S., and Yan, G. (2006) Transcriptome analysis of Deinagkistrodon acutus venomous gland focusing on cellular structure and functional aspects using expressed sequence tags. *BMC Genomics* **7,** 152